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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12P 7/64, C12N 15/00, A61K 48/00 — A1

(11) International Publication Number: WO 98/33932

(43) International Publication Date: 6 August 1998 (06.08.98)

(21) International Application Number: PCT/KR98/00020

(22) International Filing Date: 31 January 1998 (31.01.98)

(30) Priority Data:
1997/3022 31 January 1997 (31.01.97) KR

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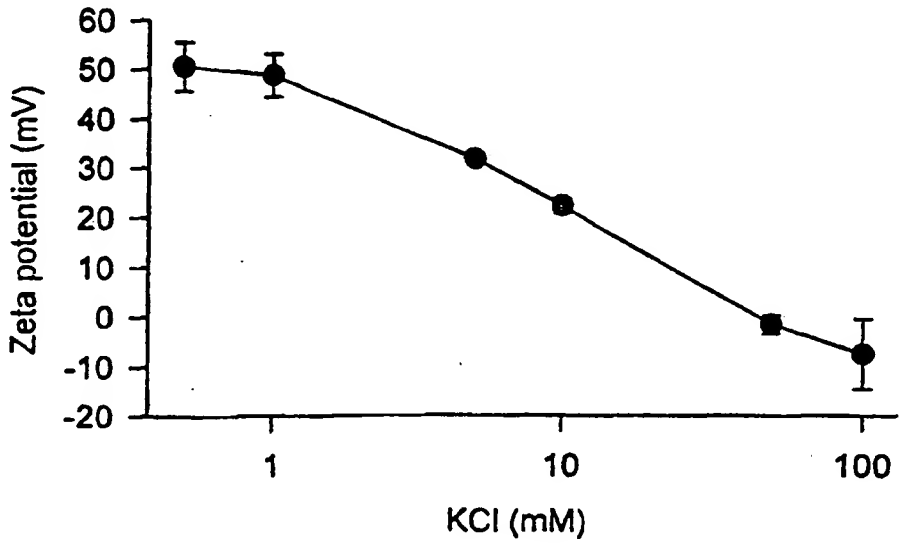
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: LIPID EMULSIONS AS GENE TRANSFECTION AGENTS AND METHOD OF PREPARING SAID EMULSIONS




(57) Abstract

An oil–in–water (o/w) type lipid emulsions and method of preparing said emulsions that can deliver genes and biologically active materials into cells are provided. The lipid emulsion of the present invention comprises (a) a combination of 2 to 30 (w/w)% of one or more oils from vegetable oils and/or triacylglycerols having 8 to 12 carbons in the hydrocarbon chains, (b) 0.01 to 20 (w/w)% of one or more emulsifiers including cationic biological surface–active agent and (c) the aqueous phase. The present invention also comprises the methods of preparing said emulsions.

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# LIPID EMULSIONS AS GENE TRANSFECTION AGENTS AND METHOD OF PREPARING SAID EMULSIONS

## TECHNICAL FIELD

The present invention relates to oil-in-water lipid emulsions used as gene transfection agents and method for preparing the emulsions. The present invention also concerns the method of transferring genes efficiently into cells by using the lipid emulsions. The lipid emulsion of the present invention comprises a combination of 2 to 30 (w/w)% of one or more oils selected from vegetable oils and/or triacylglycerols having 8 to 12 carbons in hydrocarbon chains, 0.01 to 20 (w/w)% of one or more emulsifiers including cationic biological surface-active agent, and an aqueous solution, in addition, other additives may be optionally added. The emulsions of the present invention can transfer genes into the cytoplasm. These emulsions are very stable, and can transfer genes into the cells in the presence of serum. Therefore, the emulsions of the present invention can be used in clinical gene therapy.

## BACKGROUND ART

It is critical in gene therapy to deliver genetic materials efficiently and safely to desired target cells. Many in vitro and in vivo gene transfer methods have been developed. These include DNA co-precipitation with calcium

phosphate or poly-cations and DNA microinjection. Replication-defective viral infection and particle bombardment method have also been used. Recently, cationic liposomes were developed for safe in vitro gene transfection. So far none of these methods, however, meet the requirements of both safety and efficiency for in vivo application.

A number of cationic liposomal reagents are commercially available and are widely used for gene transfection since they are able to interact strongly with both negatively charged genetic materials and plasma membranes. They have been proven to be highly effective reagents for the transfection of DNA, RNA, and oligonucleotides in a wide variety of cell lines. Unlike other available transfection agents, cationic liposomes are safe and have low cytotoxicity. They are also relatively efficient for in vitro applications. Most cationic liposomes, however, form aggregates with a high concentration of DNA, and its transfection efficiency decreases sharply in the presence of serum. These factors hamper the in vivo application of gene transfer.

An emulsion is a stable dispersion of one liquid in a second immiscible liquid, and typically, a surface-active agent is used to maintain its stability.

Emulsions made of an oil dispersed in an aqueous phase with a suitable emulsifying agent have an important part in drug delivery, as well as for delivery of parenteral nutrition. Especially, the lipid emulsions (also known as a fat emulsion or a lipid microsphere) formulated with soybean oil and

stabilized by phospholipids have been used successfully as a carrier of lipophilic drugs, since they mimic the chylomicron which is a natural fat particle that presents in the blood. A lipid emulsion could be formulated to be very stable and can be stored at room temperature for two years. For instance, parenteral emulsions have no known side-effects, even at dosage levels of 500 ml. Also, they can be easily prepared in a large quantity at low costs. Lipid emulsions are known to distribute in various tissues similar to liposomes. Therefore, the lipid emulsion can replace liposomes in many applications. It has also been reported that lipid emulsions are more stable than liposomes in the blood circulation.

## DISCLOSURE OF THE INVENTION

The present invention relates to an oil-in-water (o/w) type lipid emulsions and method of preparing the emulsions that can deliver genes and biologically active materials into cells. The lipid emulsion of the present invention comprises a combination of 2 to 30 (w/w)% of one or more oils selected from vegetable oils and/or triacylglycerols having 8 to 12 carbons in hydrocarbon chains, 0.01 to 20 (w/w)% of one or more emulsifiers including cationic biological surface-active agent, and an aqueous solution. The present invention also comprises the methods of preparing the emulsions.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

Figure 1 is a graph showing an average droplet size of the lipid emulsion, BRC 001 of the present invention as a function of the potassium chloride concentration;

Figure 2 is a graph showing an average zeta (ξ) potential of the lipid emulsion, BRC 001 of the present invention as a function of the potassium chloride concentration;

Figure 3 is an electrophoresis photograph showing a complex formation between DNA and BRC 001 of the present invention;

Figure 4 is a thin Layer Chromatography (TLC) autoradiogram showing an effect of a lipid emulsion concentration on a gene transfection efficiency in COS-1 cell line when forming the complex between DNA and BRC 001 of the present invention;

Figure 5 is a TLC autoradiogram showing an effect of a lipid emulsion concentration on a gene transfection efficiency in CV-1 cell line when forming a complex between DNA and BRC 001 of the present invention;

Figure 6 is a TLC autoradiogram showing an effect of a lipid emulsion concentration on a gene transfection efficiency in HeLa cell line when forming a complex between DNA and BRC 001 of the present invention;

Figure 7 is a TLC autoradiogram showing an effect of a lipid emulsion concentration on a gene transfection efficiency in K562 Lymphoma cell line when forming a complex between DNA and BRC 001 of the present invention;

Figure 8 is a TLC autoradiogram showing an effect of an incubation time of DNA-BRC 001 complex on a CAT gene expression;

Figure 9a is a TLC autoradiogram showing a serum effect on a CAT gene expression when Lipofectamine is used as a gene carrier in COS-1 cell line;

Figure 9b is a TLC autoradiogram showing a serum effect on a CAT gene expression when BRC 001 of the present invention is used as a gene carrier in COS-1 cell line;

Figure 10a is a TLC autoradiogram showing a serum effect on a CAT gene expression when Lipofectamine is used as a gene carrier in CV-1 cell line;

Figure 10b is a TLC autoradiogram showing a serum effect on a CAT gene expression when BRC 001 of the present invention is used as a gene carrier in CV-1 cell line;

Figure 11 is a TLC autoradiogram showing a serum effect on a CAT gene expression when pure DOTAP liposome is used as a gene carrier in COS-1 cell line;

Figure 12 is a TLC autoradiogram showing a result of a gene transfection by using commercially available liposome carriers: Lipofectin, Lipofectamine and Liofectace in COS-1 cell line;

Figure 13 is an X-gal staining photograph showing COS-1 cells after a gene transfection using Lipofectamine and BRC 001 of the present invention as carriers;

Figure 14 is an electrophoresis photograph showing that lipid carriers protect DNA against an enzyme digestion by DNAase I;

Figure 15 is an electrophoresis photograph showing a stability of a carrier-DNA complex when exposed to poly-L aspartic acid;

Figure 16 is a graph showing a comparison of a gene transfection efficiencies between emulsions prepared by different methods;

Figure 17 is a graph showing a comparison of an intranasal gene transfection efficiency between different gene carriers in Balb/C mice;

Figure 18 is a graph showing a comparison of an intramuscular gene transfection efficiency between different gene carriers in Balb/C mice;

Figure 19 is a TLC autoradiogram showing a gene expression in different organs after a systemic delivery of a DNA-BRC 001 complex via an intravenous injection;

Figure 20 is a graph showing a cell viability as a function of BRC 001 concentration of the present invention;

Figure 21a is a graph showing a size of DNA-BRC 001 complex as a function of DNA/DOTAP ratio;

Figure 21b is a graph showing a zeta potential of DNA-BRC 001 complex as a function of DNA/DOTAP ratio;

Figure 22a is a graph showing a size of DNA-BRC 001 complex of emulsions of the present invention prepared by different methods; and

Figure 22b is a graph showing a zeta potential of DNA-BRC 001 complex of emulsions of the present invention prepared by different methods.

## MODES FOR CARRYING OUT THE INVENTION

The present invention relates to an oil-in-water (o/w) type lipid emulsions that can deliver genes and biologically active materials into cells. The emulsions are composed of 2 to 30 (w/w)% of one or more oils selected from vegetable oils and/or triacylglycerols having 8 to 12 carbons in hydrocarbon chains, 0.01 to 20 (w/w)% of one or more emulsifiers, including cationic surface-active materials, and an aqueous solution, in addition, other additives may be optionally added.

Examples of the vegetable oils that may be used in the compositions of the present invention are soybean, cottonseed, olive and poppyseed oils. The cationic surface-active agents, phospholipids or non-ionic surface-active agents can be used as the emulsifiers in the composition of the present invention. Examples of the cationic surface-active agents that may be used in the compositions of the present invention are 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium chloride (DDAB) and N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). These cationic surface-active agents form complexes with DNA. The residual positive charge of the formed complex also enhances the interaction with cells. Examples of the phospholipids that may be used in the compositions of the present invention are phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, and their respective derivatives. Among them, 1,2-dioleoyl-sn-3- phosphatidylethanolamine

(DOPE) is a well-known fusogenic agent that can break the endosomal membrane and can expose the genes to the cytoplasm. Examples of the non-ionic surface-active agents that may be used in the compositions of the present invention are poloxamers (also known as pluronic: a copolymer of polyoxyethylene and polyoxypropylene), sorbitan esters (Span) and polyoxyethylene-sorbitan fat acid esters (Tween).

Additionally, the present invention may comprise of hydrophilic polymers or polymeric lipid where the hydrophilic polymers are covalently-bonded to a phospholipid. Examples of the hydrophilic polymers that may be used in the present invention are polyoxyethylene, polyethyloxazoline and polyethyleneglycol (PEG). Among them PEG and polymeric lipid enhance the sterical stability of the emulsion, and also PEG with a small molecular weight is a well-known fusogenic agent.

The compositions of the present invention may also comprise of an osmotic pressure regulator such as glycerol.

The present invention may also comprise of low-molecular weight polyethylene glycol (average molecular weight in the range of 500-1000) and fusogenic peptide, such as HA gp 41, to improve the transfection efficiency.

The present invention may also comprise of materials such as glycolipid, lipopeptide, antibody, ligand for receptors, viral protein to target

specific cells or organs.

In case the biologically active material to be deliver is a nucleic acid such as DNA, a carrier must have a positive charge to form a complex with the nucleic acid and also must be within the appropriate size range. For an in vivo application, the serum effect must also be considered. The lipid emulsion of the present invention is physically stable and has high transfection efficiency in the presence of serum (Fig. 6).

The present invention relates to the method of preparing the oil-in-water (o/w) type lipid emulsions that can deliver genes and biologically active materials into cells. The emulsions of the present invention is prepared by the oily solution containing 2 to 30 (w/w)% of one or more oils selected from vegetable oils and/or triacylglycerols having 8 to 12 carbons in the hydrocarbon chains, 0.01 to 20 (w/w)% of one or more emulsifiers including cationic surface-active agents, and the aqueous solution, in addition, other additives may be optionally added. The two solutions are prepared separately. The oily and aqueous solutions are mixed by using a well known procedure in this field.

The emulsification can be achieved by using such tools, as a stirrer, vortex mixer, homogenizer, sonicator, microfluidizer, etc.

The present invention may also comprise the method of delivering

biologically active material such as DNA into the cells by using lipid emulsions.

The method of delivering the materials is to prepare the complex of the emulsion of the present invention and the biologically active material, and to transfer the complex into the target cells. The biologically active material is selected from a group comprising of DNA, ribonucleic acid(RNA), antisense nucleic acid, ribosome, polynucleotide, oligonucleotide and other pharmaceutical drugs. The cell is selected from a group comprising of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, microphages, plant cells, animal cells, and immotalized cell lines.

When the lipid emulsion of the present invention is used as the carrier of genetic materials, it can be administered intravenously, intramuscularly, intranasally, intratracheally, subcutaneously, parenterally, by a topical administration, or direct administration to a specific organ. It can be used as a vaccine, in a diagnostic kit and for therapy.

The invention is further illustrated by the following examples.

Example 1: Preparation of the emulsion (BRC 001)

The emulsion which has components and compositions in this example is labled BRC 001 to distinguish it from the emulsions in other examples.

The lipid emulsion, BRC 001, was prepared by using the following procedure.

Example 1-1: Preparation of BRC 001 by using a homogenizer and Microfluidizer

1) Oily solution

The oily solution consisted of the following ingredients (expressed in weight %):

- 10 % soybean oil (Sigma Chemical Company, St. Louis, MO);
- 0.4 % 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Alabaster, AL);
- 0.8 % 1,2-dioleoyl-sn-glycero-3-trimethylammonium-propane (DOTAP, Avanti); and
- 0.4 % 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphoethanolamine-N-[poly (ethylene glycol) 2000] (PEG2000 PE, Avanti).

2) Aqueous solution

Two point two five (2.25) % glycerol (Sigma) quantity sufficient with distilled and deionized water (DDW) to 100%.

The two solutions were heated at 70°C and stirred separately to completely solubilize the components. The emulsion was prepared by mixing the two solutions with a high-speed homogenizer (T-25-Ultra-Turrax, S25-18G, IKA Werke, Janke & Kunkel GmbH & Co KG, Germany) at 8000 rpm for 10 min. The mixture was passed 10 times through a Microfluidizer® (Microfluidics Co., Newton, MA) with an exit air pressure of 80 psi. The emulsion was kept at 4°C until further use.

The emulsion was diluted 300 times with DDW (> 18 MΩ) to measure its droplet size and zeta potential. The zeta potential is the potential of the droplets in the emulsion at the surface of shear. The average droplet size (diameter) and the zeta potential of the emulsion measured by a particle Electrophoresis Analyzer, Malvern Instruments Limited, England, and were 185 ± 13 nm and 56.3 ± 3.2 mV (n=3), respectively.

Example 1-2: Preparation of BRC 001 by using a sonicator.

The oily and aqueous solutions were prepared as in example 1-1. The two solutions were heated at 70°C separately to completely solubilize the components. The emulsion was prepared by sonicating the mixture by using a probe-type sonicator (High intensity ultrasonic processor, 600 W model) for 2 min. The emulsion was diluted 300 times with DDW to measure its size and surface potential. The average droplet size (diameter) and the zeta potential of the emulsion were 237.9 ± 35.9 nm and 49.9 ± 0.9 mV (n=3), respectively.

The emulsion was kept at 4°C until further use.

Example 1-3: Preparation of BRC 001 by using a sonicator.

The oily solution of example 1-1 was prepared, except for the use of the soybean oil and was completely dissloved with the addition of chloroform. The chloroform was subsequently removed by using a rotary evaporator (Buchi Rotavapor, Switzerland) to form a lipid film around an inside wall of a round-bottomed flask. An identical aqueous solution as in example 1-1 was added to the lipid film. The solution was sonicated to form a liposome solution. Soybean oil was added to this liposome solution and further sonicated for 2 minutes to formulate the emulsion. The emulsion was diluted 300 times with DDW (> 18 MΩ) to measure its size and surface potential. The average droplet size (diameter) and the zeta potential of the emulsion were 272.8 ± 25.9 nm and 53.4 ± 2.2 mV (n=3), respectively. The emulsion was kept at 4°C until further use.

Example 2: Preparation of the emulsion other than BRC 001

Example 2-1:Variation of the composition

The lipid emulsions of various compositions were prepared by using the method as in example 1-1 as follows:

**Table 1**

| Emulsion | Soybean oil | DOPE | DOTAP | $PEG_{200}$ PE | $PEG_{5000}$ PE | Cholesterol | Glycerol | DDW |
|---|---|---|---|---|---|---|---|---|
| A | 10% | 0.4% | 0.8% | - | - | - | - | QS to 100% |
| B | 10% | 0.4% | 0.8% | - | - | - | 2.25% | QS to 100% |
| C | 10% | 0.4% | 1.2% | - | - | - | 2.25% | QS to 100% |
| D | 10% | 0.4% | 0.8% | 0.4% | - | - | 2.25% | QS to 100% |
| E | 10% | 0.4% | 0.8% | - | 0.04% | - | 2.25% | QS to 100% |
| F | 10% | 0.4% | 0.8% | - | - | 0.08% | 2.25% | QS to 100% |
| G | 20% | 0.8% | 1.6% | - | 0.08% | - | 2.25% | QS to 100% |
| H | 5% | 0.2% | 0.4% | 0.02% | - | - | 2.25% | QS to 100% |
| I | 30% | 1.2% | 2.4% | 0.12% | - | - | 2.25% | QS to 100% |

The average droplet size and the zeta potential of the emulsion were measured by using the same method as in example 1-1 and were ca. 200-300 nm and 50 mV, respectively.

Example 2-2: Variation of the core oils in the emulsion

The lipid emulsions were prepared by using the method as in example 1-3 with different core oils other than soybean oil. The average droplet size and the zeta potential of the emulsion were measured by using the same method as in example 1-1 (Table 2). The constituents and composition other than soybean oil were identical to BRC 001 shown in example 1.

**Table 2**

| Emulsion | Core oil | Size (nm) | Zeta potential (mV) |
|---|---|---|---|
| J | 10 % Castor oil | 332.0 ± 25.0 | 55.3 ± 1.3 |
| K | 10 % Cottonseed oil | 299.5 ± 17.3 | 51.8 ± 1.3 |
| L | 10 % Tricaprylin | 265.0 ± 35.3 | 51.9 ± 0.6 |

Tricaprylin is a triacylglycerol that has 8 carbons in the hydrocarbon chain.

Example 2-3: Variation of other components in the emulsion

The lipid emulsions were prepared by changing other constituents by using the method as in example 1-2. Pluronic F68 (BASF Corp., Parsippany NJ), which is a poloxamer, is an emulsifier and dissolves well in water. GM1 ganglioside (Sigma) is a glycolipid and also dissolves well in water. Therefore, they were dissolved in water to prepare the emulsions. The average droplet size and the zeta potential of the emulsion were measured by using the same method as in example 1-1 (Table 3).

**Table 3**

| Emulsion | Oily solution | Aqueous solution | Size (nm) | Zetapotential (mV) |
|---|---|---|---|---|
| M | 10 % Soybean oil<br>0.4 % DOPE<br>0.8 % DOTAP | 0.04 % Pluronic F68<br>2.25 % glycerol | 293.1 ± 2.3 | 61.8 ± 0.6 |
| N | 10 % Soybean oil<br>0.4 % DOPE<br>0.8 % DDAB<br>0.4 % $PEG_{2000}PE$ | 2.25 % glycerol | 258.1 ± 2.3 | 60.0 ± 2.0 |
| O | 10 % Soybean oil<br>0.4 % DOPE<br>0.8 % DOTAP<br>0.4 % $PEG_{2000}PE$ | 2.25 % PEG1000 | 232.1 ± 1.3 | 60.1 ± 2.0 |
| P | 10 % Soybean oil<br>0.4 % DOPE<br>0.8 % DOTAP<br>0.4 % $PEG_{2000}PE$ | 0.02 % GM1 ganglioside<br>2.25 % glycerol | 57.3 ± 0.2 | 251.3 ± 3.8 |

Example 3: Stability test

Example 3-1: Stability of emulsions in PBS solution

Stability of the emulsions prepared as in examples 1 and 2 were tested in phosphate buffered saline (PBS) solution. Electrostatic repulsion is one of

the important factors in stabilizing the emulsions since BRC 001 has an excess positive charge. The positive charge can be shielded in PBS, which is isotonic to serum, due to the presence of the counter ions. Therefore the cationic emulsions can become less stable in PBS or in the presence of serum. Therefore, it is an important to make certain that the carrier is stable in PBS. The average droplet size and the absorbance at 450 nm (Lambda 18 UV-VIS Spectrophotometer, Perkin Elmer, Norwalk, CT) of some of the emulsions prepared in examples 1 and 2 were measured (Table 4).

**Table 4**

| Size (nm) at time t | BRC 001 | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| t = 0 h | 180.5 | 329.3 | 367.1 | 250.3 | 222.2 | 265.0 | 345.5 |
| T = 2 h | 185.9 | 497.2 | 554.3 | 618.2 | 431.1 | 312.7 | 1520.2 |

The average droplet size change of the emulsions after diluting them 300 time in PBS solution.

Example 3-2: Stability of emulsions in PBS solution

The BRC 001 prepared as in example 1-1 was sterilized by autoclaving at 120° C for 20 min. The sterilized emulsions were diluted 300 times in DDW for the size and zeta potential measurements (Table 5).

**Table 5**

| | Before autoclave | After autoclave |
|---|---|---|
| Size (nm) | 185.2 ± 13.0 | 176.4 ± 6.1 |
| Zeta potential (mV) | 58.1 ± 4.5 | 62.5 ± 6.7 |

Example 3-3: Stability of emulsions in KCl solutions

Stability of the BRC 001 as prepared in example 1-1 was tested in various concentrations of potassium chloride (KCl) solutions. The size and the zeta potential of the BRC 001 were measured after diluting the emulsion 300 times in KCl solutions of different concentrations. The results are shown in Figures 1a and 1b, respectively. The size of the emulsion did not change for 24 h after the dilution. The emulsion was stable in the KCl solutions of high concentrations. This result shows that the steric repulsion could provide the emulsion stability. The steric repulsion that polyethylene glycol moiety of the $PEG_{2000}PE$ provides is one of the most important emulsion stabilizing factor. It is particularly important to maintain emulsion stability when the charge repulsion could not act, especially when the DNA-carrier complex is formed or in the presence of serum. The conventional emulsions that use egg phosphatidylcholine as an emulsifier is often unstable in high salt concentration. The emulsion of the the presentinvention, however, overcame the stability problem in high salt concentrations.

Example 3-4: Percent turbidity measurement

The stability of the BRC 001 prepared by using the method in examples 1-1 and 1-2 were compared by measuring the percent turbidity. The emulsions were diluted 300 times in PBS buffer solution before measuring the absorbance at 450 nm (n=3). The turbidity (Turbidity=10Abs) did not change from the initial value as a function of time (Fig. 2). Since the transfection agents must be used in isotonic conditions, they must be stable in PBS.

Example 4: Cell culture and isolation of plasmid DNA

Cell culture

COS-1 cells (kidney, SV40 transformed, African green monkey) were grown in RPMI1640 supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells (Human cervical carcinoma) were cultured in DMEM supplemented with 10 % fetal bovine serum, 10 mM non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Isolation of plasmid DNA

A plasmid DNA containing the chloramphenicol acetyl transferase driven by a SV40 (pSV-CAT) or a CMV (pCMV-CAT) promoters or a plasmid where a CMV promoter drove the expression of β-galactosidase with a nuclear localization signal (**pCMV-gal**) as reporter genes was purchased from Promega, WI, U.S.A. The plasmid was amplified in the Escherichia coli (E.Coli)

HB101 strain and purified by an alkaline lysis technique according to Quiagen (Quiagen Inc., Chatsworth, CA) mega-kit preparation protocol as described in the manufacturer's instruction.

Example 5: DNA-BRC 001 complex formation

The complex formation between pSV-CAT gene and BRC was observed by using a gel electrophoresis technique. The pSV-CAT gene was diluted to a concentration of 5 $\mu$g per 100 $\mu$l in serum free RPMI. Various concentrations of lipid emulsion solutions diluted in 100 $\mu$l of RPMI 1640 medium were added to the DNA solutions and incubated for 20 to 60 minutes at room temperature. As a control, Lipofectamine® also diluted in the same medium ( 20 $\mu$g / 100 $\mu$l) was added to the DNA solution. The DNA-liposome complexes were formed after a 20 minute incubation at room temperatures according to the manufacturer's protocol. The size and zetapotential changes are shown in Tables 6 and Table 7, respectively. Unlike Lipofectamine, the BRC 001's size nor zeta potential change before or after the complex formation with DNA. This suggest that the mechanism for the BRC 001-DNA complex formation is different from that for Lipofectamine-DNA.

**Table 6**

Size change of the Carrier-DNA complex in RPMI 1640 solution

| Incubation time (h) | BRC 001 (nm) | BRC 001-DNA (nm) | Lipofectamine (nm) | Lipofectamine-DNA (nm) |
|---|---|---|---|---|
| 0 | 174.9 ± 2.4 | 170.7 ± 9.2 | 183.1 | 306.9 |
| 1 | 172.1 ± 4.3 | 171.7 ± 7.8 | 151.4 | 771.7 |

BRC 001 (n=3, average ± standard deviation)

**Table 7**

Zeta potential change of the Carrier-DNA complex in DDW

| | Carrier only | Carrier incubated with DNA for 1 h |
|---|---|---|
| BRC 001 | 62.4 ± 6.6 | 59.0 ± 2.8 |
| Lipofectamine | 61.9 ± 3.7 | 21.4 ± 5.7 |

Five (5) μg of pSV-CAT plasmid DNA mixed with 1, 3, 6, 9, 12, 15 and 20 μl each of BRC 001 of the present invention were mixed in 100 μl of serum free RPMI 1640 media. Ten (10) μl of Lipofectamine was used instead of the BRC 001 as a control. The mixtures were incubated for 1 h at room temperature for the complex formation. Twenty (20) μl of the samples were loaded in agarose gel for the electrophoretic analysis in Tris acetate-EDTA buffer solution at pH 8.0. The DNA-carrier complex and free DNA were

visualized by observing ethidium bromide fluorescence at 30 min after the experiment (Fig. 3). Lipofectamine formed the complex with DNA completely without a trace of free DNA. In case of BRC 001, the complex formation depended on the BRC 001 concentration.

Example 6: Gene transfection and CAT analysis

Example 6-1: Gene transfection efficiency in COS-1 cell line

COS-1 cells were seeded at 5 x $10^5$ cells in a 60 mm-diameter dish one day prior to the transfection. Five (5) $\mu$g of pSV-CAT plasmid DNA were mixed with 0.02, 0.033, 0.1, 1, 3, 6, 9, 12, 15 and 20 $\mu$l each of the BRC 001, respectively. The mixtures were diluted in 100 $\mu$l of serum free RPMI 1640 medium. Ten (10) $\mu$l of Lipofectamine was used instead of the BRC 001 as a control. The mixtures were completely shaken three times during an one-hour incubation period. After cleaning the COS-1 cells with serum free RPMI 1640, 800 $\mu$l serum free media and carrier-DNA mixture were added to them. Additional 3 ml serum free RPMI 1640 was added to each well. After a 4 h incubation at 37° C in a 5 % carbon dioxide incubator, the cells were washed with serum free RPMI 1640 to remove the remaining carrier-DNA complex in the solution. The cells were incubated in RPMI 1640 containing 10% serum for 48 hs. The transfected cells were harvested after the incubation. A chloramphenicol acetyl transferase (CAT) enzyme activity of the cell lysates was determined using an autoradiography. Cells were washed

twice with PBS, were harvested with 500 $\mu l$ of 0.25 M Tris-Cl (pH 7.8), and were lysed by means of three freeze-thaw cycles. The protein concentration in supernatant was measured with a micro BCA protein assay reagent kit (Pierce, USA), and equal amounts of homogenate were assayed as described by the manufacturer. The results indicate that the BRC 001 of the present invention can transfer DNA into COS-1 cells in a concentration-dependent fashion.

Example 6-2: Gene transfection efficiency in CV-1 cell line

All the procedures were identical as in example 6-1 except that CV-1 cells and pCMV-CAT plasmid DNA (from E.Coli) were used instead the of COS-1 and pSV-CAT, respectively. Gene transfection was assayed by CAT analysis (Fig.5). The results indicate that the BRC 001 of the present invention can transfer DNA into CV-1 cells in a concentration-dependent fashion.

Example 6-3: Gene transfection efficiency in HeLa cell line

All the procedures were identical as in example 6-1 except that HeLa cells and pCMV-CAT plasmid DNA (from E Coli) were used instead of the COS-1 and pSV-CAT, respectively. Gene transfection was assayed by CAT analysis (Fig.6). The results indicate that the BRC 001 of the present invention can transfer DNA into HeLa cells in a concentration-dependent fashion.

Example 6-4: Gene transfection efficiency in K562 cell line

All the procedures were identical as in example 6-1, except that K562 lymphoma cells and pCMV-CAT plasmid DNA (from E.Coli) were used instead of the COS-1 and pSV-CAT, respectively. Gene transfection was assayed by CAT analysis (Fig.7). The results indicate that the BRC 001 of the present invention can transfer DNA into K562 cells in a concentration-dependent fashion.

Example 7: Effect of incubation time on transfection efficiency

All the procedures were identical as in example 6, except that the incubation time varied from 20 mins to 2 hs for the complex formation between DNA and the carriers (Lipofectamine and BRC 001). The transfection efficiency was maximal with a 20 min incubation for the Lipofectamine-DNA complex (Fig. 8). (Longer or shorter than 20 min incubation resulted in a big decrease in transfection efficiency.) The BRC 001 and DNA seem to form a stable complex with good transfection efficiency during this incubation period.

Example 8: Effect of serum on transfection efficiency

Example 8-1: Effect of serum on transfection efficiency in COS-1 cell line

To test the serum effect on DNA transfection efficiency, carrier-DNA

complexes were prepared in RPMI 1640 without serum. These complexes were applied to the cells in RPMI medium containing various concentrations of FBS (up to 90 %) for 4 hours and the medium was changed with fresh RPMI with 10 % FBS. The transfected cells were harvested in 48 hours and analyzed as in example 6-1 (Fig. 9). In the presence of serum, the transfection efficiency decreased sharply for the Lipofectamine-DNA complex (Fig. 9A). For the BRC 001-DNA complex, however, the transfection efficiency did not decrease with up to 90 % serum (Fig. 9B). The result indicates that BRC 001 can be used as a stable gene carrier for in vivo applications.

Example 8-2: Effect of serum on transfection efficiency in CV-1 cell line

Identical procedure was used as in example 8-1 for the CAT analysis, except that the transfection was performed as in example 6-2 (Fig. 10). In the presence of serum, the transfection efficiency decreased sharply for the Lipofectamine-DNA complex (Fig. 10A). For the BRC 001-DNA complex, however, the transfection efficiency did not decrease with up to 90 % serum in CV-1 cell line (Fig. 10B).

Example 8-3: Effect of serum on transfection efficiency of pure DOTAP liposome in COS-1 cell line

All the procedures were identical as in example 6-1 except, that pCMV-CAT plasmid DNA (from E.Coli) was used instead of the pSV-CAT.

The CAT analysis was performed as in example 8-1 (Fig. 11). A liposome was prepared with the emulsifiers as in example 1 without soybean oil and labled BRC 002. A pure DOTAP liposome was also prepared by sonication. A pure DOTAP liposome-DNA complex was applied to the cells in the RPMI medium containing various concentrations of FBS (up to 90 %) for 4 hours. The BRC 001-DNA complex in serum free media (BRC 001-SFM), and in the media containing 50 % serum (BRC 001- 50 %serum) and the BRC 002 liposome-DNA complex in serum free media (BRC 002) were used as controls. The pure DOTAP liposome did not show transfection efficiency in the presence of higher than 20 % serum concentration.

Example 9: X-gal staining

Each of 5 $\mu$g pSV-β-gal was mixed to 10 $\mu$l of Lipofectamine and BRC 001 of the present invention for the transfection in COS-1 cells as in example 6. The cells were harvested after 48 hours of incubation. The cells were washed twice with 5 ml each of PBS and fixed with a mixture of 1.8 % formaldehyde and 2 % glutaraldehyde. They were incubated subsequently for 16 hours at 37° C with 0.313 $\mu$l of 40 mg/ml X-gal in DMSO dissolved in 12.5 ml of PBS (pH 7.8). The blue staining of nuclei that was observed by an inverted light microscopy indicates that both Lipofectamine and BRC 001 transfered genes into cells (Fig. 12).

Comparative example 1

Transfection efficiency was qualitatively measured with commercially available liposomes, Lipofectin, Lipofectace and Lipofectamine, by CAT analysis in COS-1 cell line as described in example 6. The Lipofectamine showed the highest transfection efficiency among the liposome carriers (Fig. 13).

Example 10: Gene transfection efficiency in HepG2 cell line

All the procedures were identical as in example 6-1, except that HepG2 cells and that pSV-CAT and pSV-β-gal plasmid DNA were used. The BRC 001 of the present invention can transfer DNA into HepG2 cells in a concentration-dependent fashion.

Example 11: Protection of DNA against enzyme digestion by DNAase I

The BRC 001-DNA (pSV-CAT) and Lipofectamine-DNA complex were incubated for 1 h at room temperature. Zero point five (0.5) or 1.0 unit each of DNAase I were added to the complex and incubated for 30 min at 37° C. Two (2) $\mu$l of the Lipofectamine or BRC 001 and 1 $\mu$g of DNA were used for the experiment (Fig 14). Lanes 1 and 2 show the DNA size marker and DNA in the absence of DNAase, respectively. DNA-Lipofectamine complex with 0.5 (Lane 4) and 1.0 (Lane 5) unit of DNAase I and DNA-BRC 001 complex with

0.5 (Lane 7) and 1.0 (Lane 8) unit of DNAase I are also shown. A naked DNA was digested by DNAase I. However, DNA that formed complex with the carriers were protected by the Lipofectamine and by BRC 001.

Example 12: Protection of DNA against PLAA

The DNA complexes were formed with the Lipofectamine and BRC 001 as in example 11. Different concentrations of poly-L-aspartic acid (PLAA) were added to the complex. Since PLAA is an anionic polymer, it could dissociate the anionic DNA from the complex. Therefore, the dissociation of DNA from the complex in the presence of PLAA is an indicator for the strength of the complex. DNA was dissociated from the Lipofectamine-DNA complex at a low PLAA concentration. On the other hand, the BRC 001-DNA complex remained stable in the PLAA concentration range studied.

Example 13: Comparison of transfection efficiencies with and without serum by using different preparation methods

The BRC 001 were prepared by using the procedures as in examples 1-1 and 1-3. The transfection efficiencies of the BRC 001- DNA (pCMV-β-gal plasmid) were compared in the presence and in the absence of serum (Fig. 16). All other procedures were identical as in example 11. The Lipofectamine was used as a control. The transfection efficiency decreased greatly in the presence of serum in case of the Lipofectamine. On the other hand, the BRC

001 showed almost identical efficiency with or without serum. The BRC 001 by sonication (**so**, as in example 1-3) showed higher transfection efficiency than that by microfluidization (**mf**, as in example 1-1) by a factor of ca. 1.5 to 1.8 whether serum was the presentor not.

Example 14: In vivo experiment

The complex was prepared with 100 $\mu$l of BRC 001 and 50 $\mu$g of pCMV-CAT plasmid DNA having the same conditions as example 11. The final volume of the solution was adjusted to 160 $\mu$l with PBS. The 40 $\mu$l of solutions were delivered via intranasal route to the Balb/c mice for four times. The mice were sacrificed 48 hours after the final administration. A CAT assay was performed from the nasal extracts (Figure 17). The mice that received naked DNA or the complex with liposomes (Lipofectamine; L/P or Lipofectin; L/F) has transfection efficiency of lower than 0.4 to 0.5. However, the mice that received the BRC-DNA complex showed high transfection efficiency (1.0).

Example 15: In vivo experiment

The complex was prepared as in example 14 having the same conditions as example 11. The 40 $\mu$l of solutions were delivered via intramuscular injection in the thigh region of the Balb/c mice four times. The mice were sacrificed at 48 hours after the final administration. The CAT assay was performed from the muscle extracts at the site of injection (Figure 18).

The transfection efficiency for the mice that received the BRC 001 was almost twice than others used in the experiment.

Example 16: Systemic delivery via intravenous injection (In vivo experiment)

Ten (10) or 20 $\mu$l of the BRC 001 and 100 $\mu$g of pCMV-CAT plasmid DNA were mixed to form a complex. The complex was injected though a tail vein of an ICR mouse of ca. 30 g weight. The mouse was sacrificed 52 hours after the injection, and each organ (heart, liver, lung, kidney and spleen) was obtained for analysis. The CAT analysis was performed for the organ extracts as in example 6 (Fig. 19). The result indicates that the BRC 001 of the present invention can deliver genes.

Example 17: Cytotoxicity

Live cells can be quantified by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay. A tetrazolium ring opening reaction can take place only in the presence of the live cells with active mitochondria. This yellow-to-purple reaction can be quantified by using the scanning multiwell spectrophotometer (ELISA reader).

The BRC 001 of the present invention was prepared by the microfluidization (as in example 1-1) and by the sonication (1-3). The COS1 cells were harvested from exponential phase culture by trypsinization, counted

and plated in 96-well microplates. A concentration of 5000 cells/well was found suitable. Plates were incubated at 37° C for 1 day under a humidified atmosphere. The BRC 001 was added to the cells in different concentrations and incubated for 24 hours. After exposure with a lipid emulsion, the cells were washed twice with PBS. MTT was dissolved in PBS, filter-sterilized and stored at 4° C until further experiment. Two hundred (200) μl of fresh media and 50 μl of 0.5 % MTT solution were added to each well and incubated for 4 hours at 37° C to allow intracellular metabolization. Formazan crystals were dissolved by adding 50 μl of dimethyl sulfoxide (DMSO). Twenty five (25)μl of Sorensen's glycine buffer was added. The absorbance was measured at 570 nm on an Elisa reader [SOFTmax® PRO (Molecular Devices corporation, California, U.S.A.)]. The result indicates that more than 60 % of the COS-1 cells are alive even in high BRC 001 concentrations (Fig. 22).

Example 18: BRC 001-DNA complex

Effects of DNA to the carriers and to their characteristic were tested.

Size measurement

Solution A: the BRC 001 was diluted with RPMI 1640 media to prepare a final volume of 100 μl.

Solution B: 2.5 μg of DNA (pSV-CAT or pCMV CAT) was diluted in 100 μl RPMI 1640 media.

Solutions A and B were mixed and incubated at room temperature for 30 mins. After the incubation, RPMI 1640 media was added to a total volume of 2 ml to measure the emulsion droplet size (Fig. 21a).

Zeta potential measurement

The sample preparation method is identical to the size measurement except that DDW was used in instead of the RPMI 1640 to the dilute BRC 001 and DNA (Fig. 21b). Conventional liposome and polymer gene carriers formed aggregate with DNA resulting bigger droplets of emulsions. The transfection efficiency decreases dramatically as these aggregates are formed. However, the size and distribution of the lipid emulsion of the present invention did not change in the presence of high DNA concentration. Therefore, the emulsion of the present inventioncould be used as gene carriers at high DNA concentrations.

Example 19: Characteristic of BRC 001-DNA complex according to preparation methods

The BRC 001 emulsions were prepared by sonication (as in example 1-3) by microfluidization (as in example 1-1) to produce BRC 001-DNA (pCMV-CAT) complexes at a cation to anion ratio of 16. The size (Fig. 22a) and the zetapotential (Fig. 22b) measurements were performed as in example 18. The emulsions with the two different preparation showed similar size and

zeta potential values. It is recommended that the sonication method is suitable to produce small quantities of emulsion whereas the microfluidization is acceptable for the production of large quantities of emulsions.

5

## CLAIMS

1. An oil in water lipid emulsions comprising:

a) 2 to 30 (w/w)% of one or more vegetable oils and/or triacylglycerols having 8 to 12 carbons;

b) 0.01 to 20 (w/w)% of one or more emulsifiers, including cationic surface-active agent; and,

c) water quantity sufficient to 100% w/w.

2. The emulsion according to claim 1, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

3. The emulsion according to claims 1 or 2, wherein the emulsifier further comprises and is selected from a group comprising of a phospholipid and a non-ionic surface-active agent.

4. The emulsion according to Claims 1 or 2, wherein the cationic surface-active agent of step b) is selected from a group comprising of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium chloride (DDAB), N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTMA), and other cationic phospholipid.

5. The emulsion according to claims 1 or 2, further comprising of

a glycerol or fusogenic peptides.

6. The emulsion according to claims 1 or 2, further comprising and selected from a group comprising of a glycolipid, lipopeptide, antibody, ligand for the receptors, and viral protein to target specific cells or organs.

7. The emulsion according to claim 2, wherein the hydrophilic polymer is selected from a group comprising of a polyoxyethylene, polyethyloxazoline and polyethyleneglycol.

8. The emulsion according to claim 3, wherein the phospholipid is selected from a group comprising of a phosphatidylcholine, phosphatidylethanolamine, 1,2-dioleoyl-sn-3- phosphatidylethanolamine (DOPE), phosphatidylserine and its derivatives.

9. The emulsion according to claim 3, wherein the non-ionic surface active agent is selected from a group comprising of a poloxamer, sorbitan ester (Span), and polyoxyethylene-sorbitan fat acid ester (Tween).

10. A process of preparing an oil in water lipid emulsion comprisng:

1) preparing an oily solution comprising of vegetable oils and/or triacylglycerols and emulsifiers; and

2) mixing the oily solution with water, whereby the emulsion is obtained.

11. The process according to claim 10, wherein the process step of 1) further comprises of preparing an aqueous solution containing 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

12. The process according to claim 11, wherein the process of step 2) further comprises a process of preparing an aqueous solution containing glycerol.

13. The process of forming a complex between a biologically active material such as DNA and an oil-in-water lipid emulsion, and of transferring the complex into a cell.

14. The process according to claim 13, wherein the biologically active material is selected from a group comprising of DNA, ribonucleic acid (RNA), antisense nucleic acid, ribosome, polynucleotide, oligonucleotide, and other pharmaceutical drugs.

15. The process according to claim 13, wherein the cell is selected from a group comprising of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, macrophages, plant cells, animal cells, and immotalized cell lines.

16. The process according to claim 13, wherein transferring of the emulsions into the cells is via intravenous, intramuscular, intratracheal, intranasal, subcutaneous, parenteral or topical administration or through direct administration to a specific organ.

5

FIG. 1A

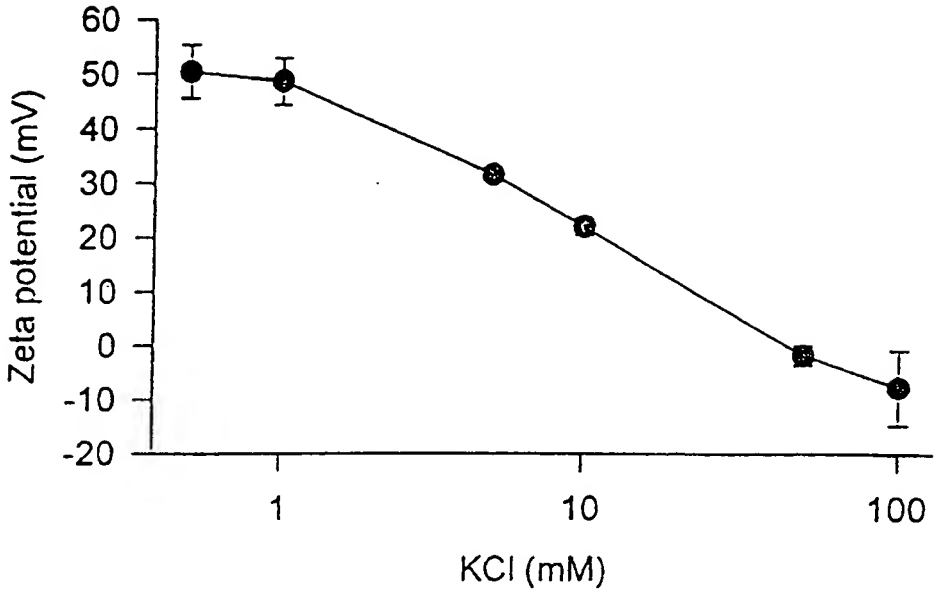

60
50
40
30
20
10
0
-10
-20
Zeta potential (mV)
1
10
100
KCl (mM)

FIG. 1B

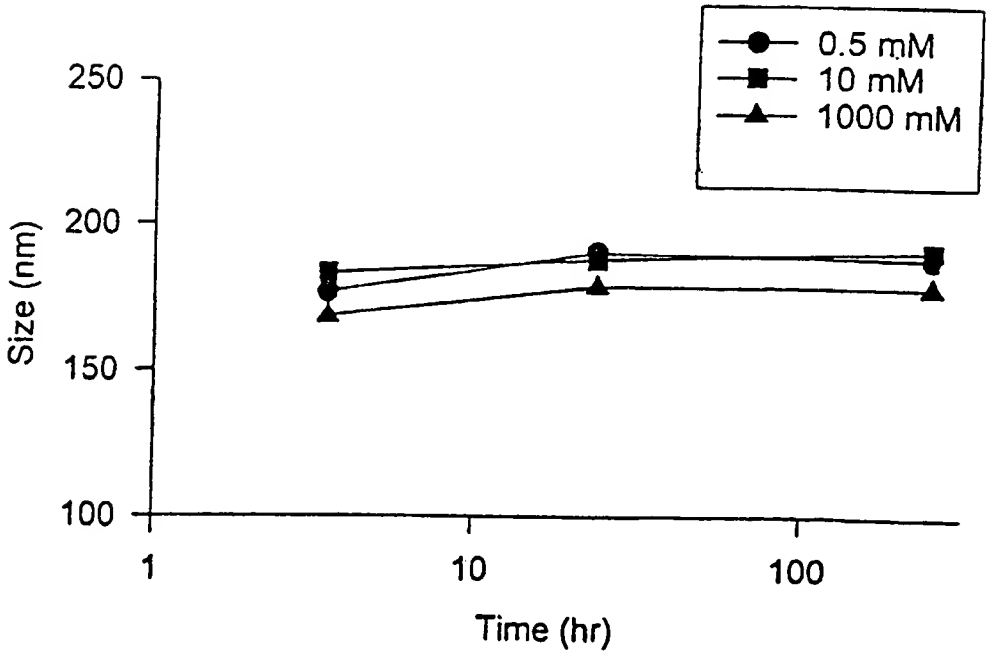

0.5 mM
10 mM
1000 mM
250
200
150
100
Size (nm)
1
10
100
Time (hr)

FIG.2

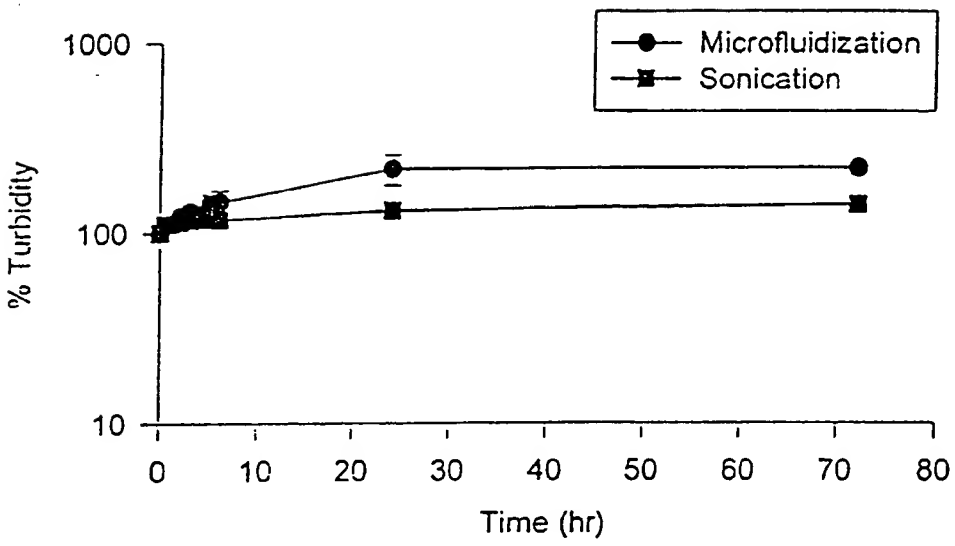

Microfluidization
Sonication
1000
100
10
% Turbidity
0
10
20
30
40
50
60
70
80
Time (hr)

FIG.3

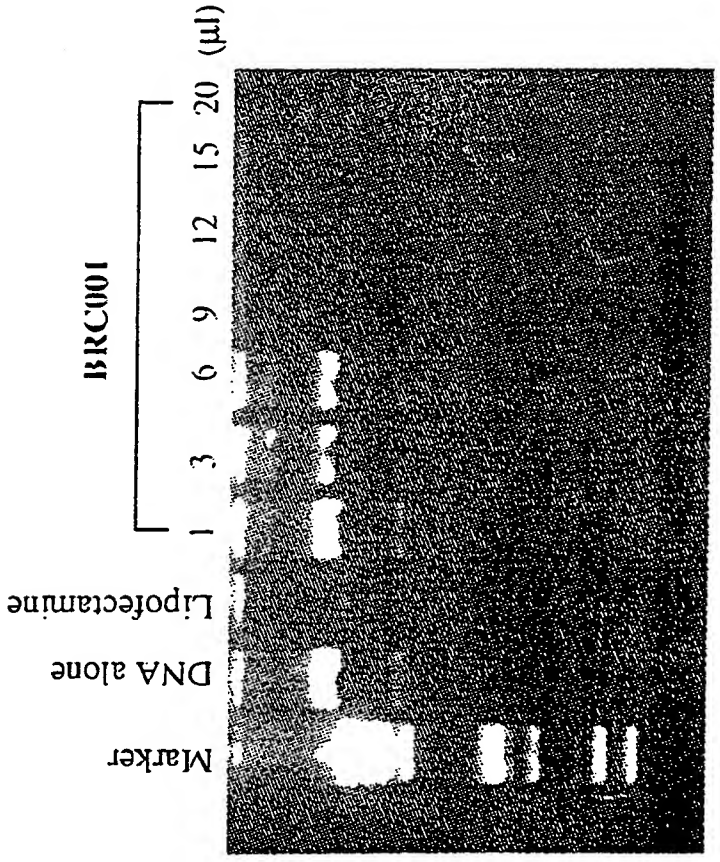


FIG.4

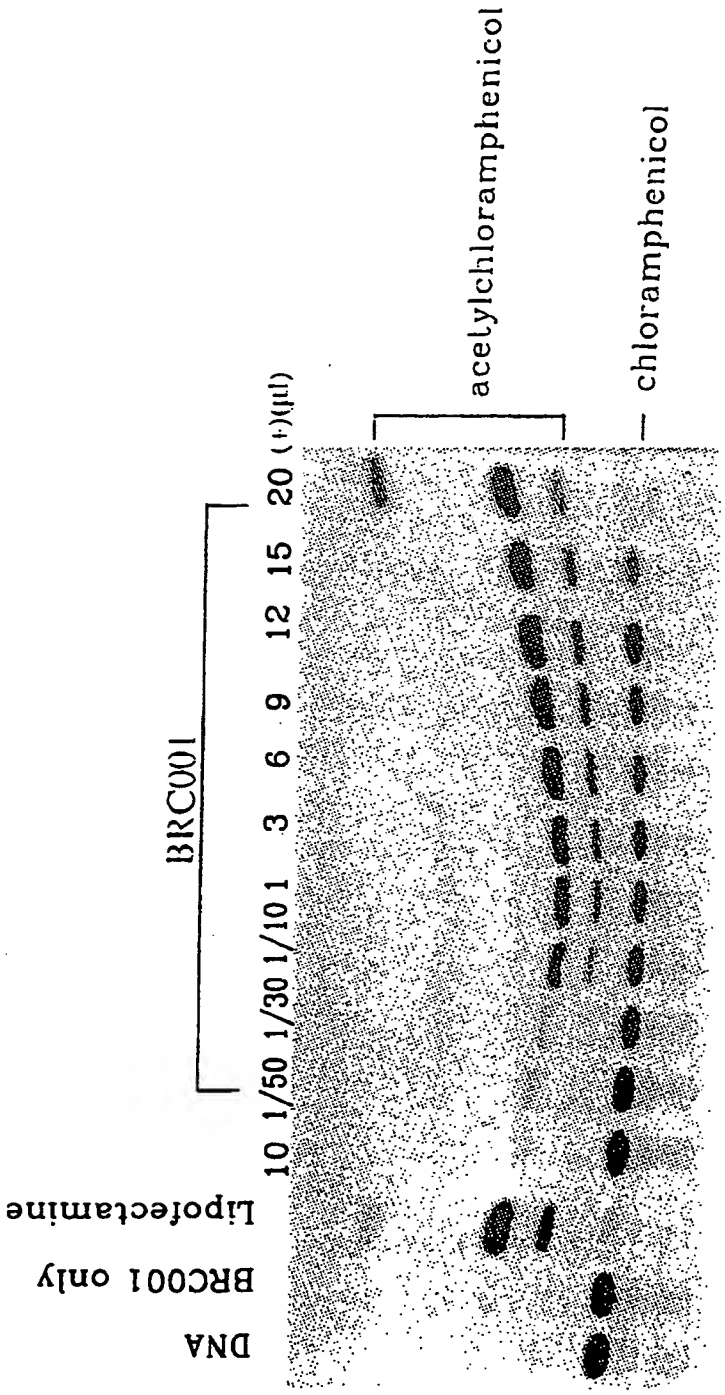

acelylchloramphenicol
chloramphenicol
BRC001
DNA
BRC001 only
Lipofectamine
10 1/50 1/30 1/10 1 3 6 9 12 15 20 (μl)

FIG.5

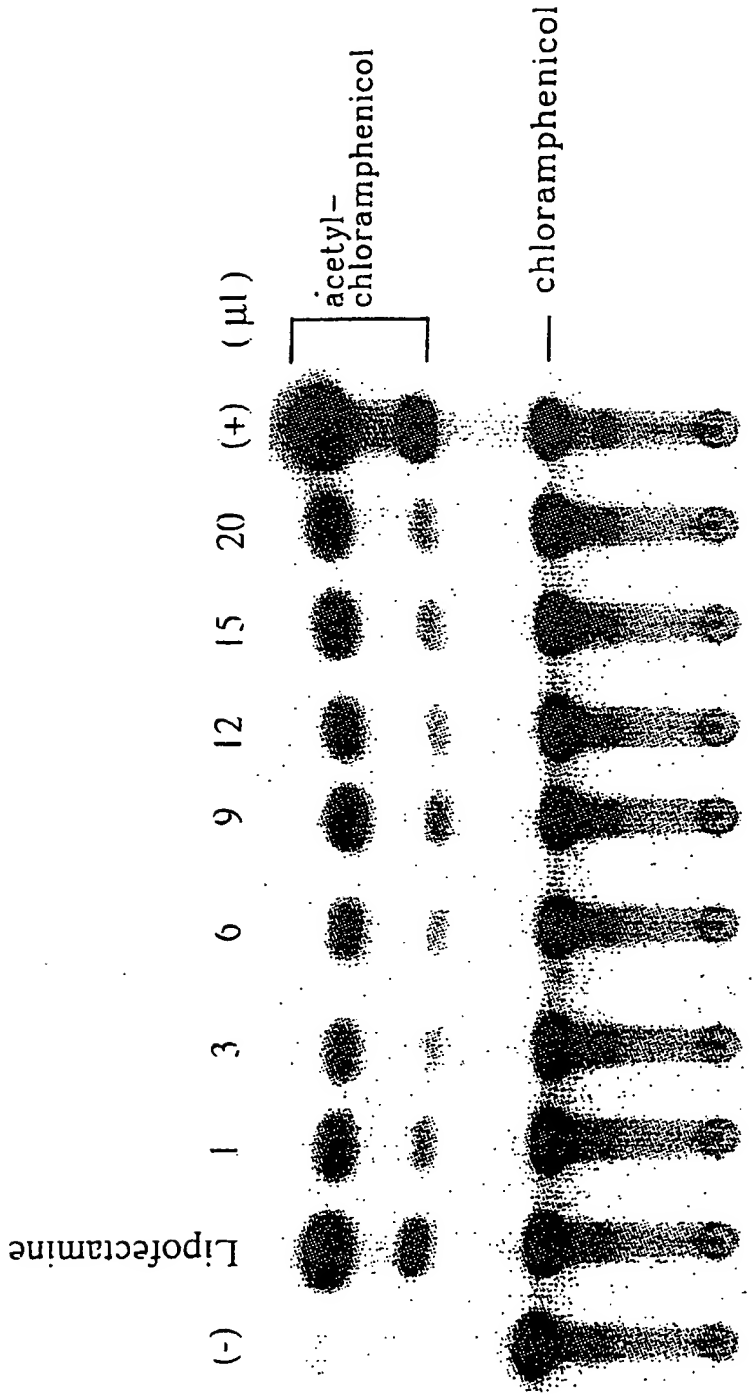
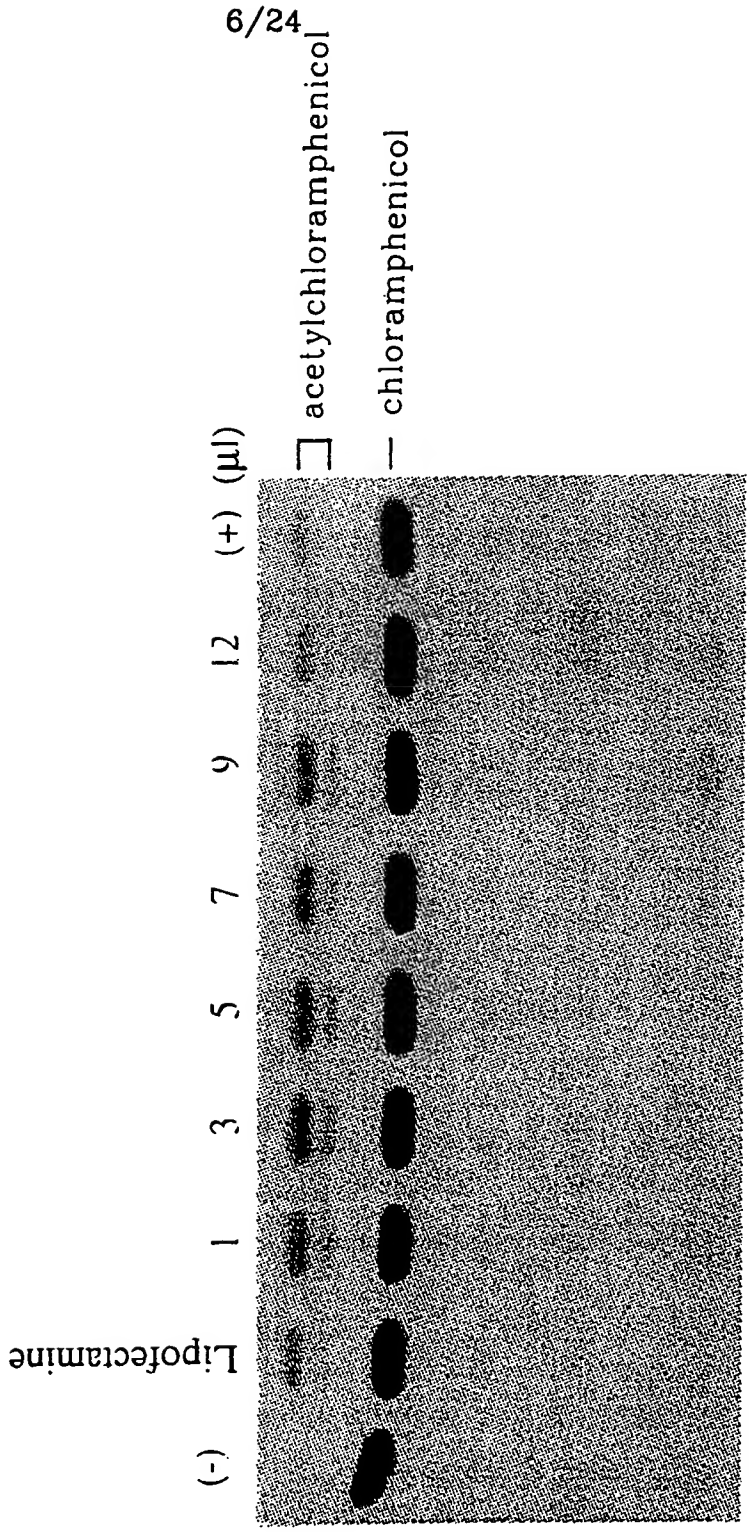

acetyl-chloramphenicol
chloramphenicol
(μl)
(+)
20
15
12
9
6
3
1
Lipofectamine
(-)

FIG.6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chloramphenicol
(μl)
(+)
12
9
7
5
3
1
Lipofectamine
(-)

FIG. 7

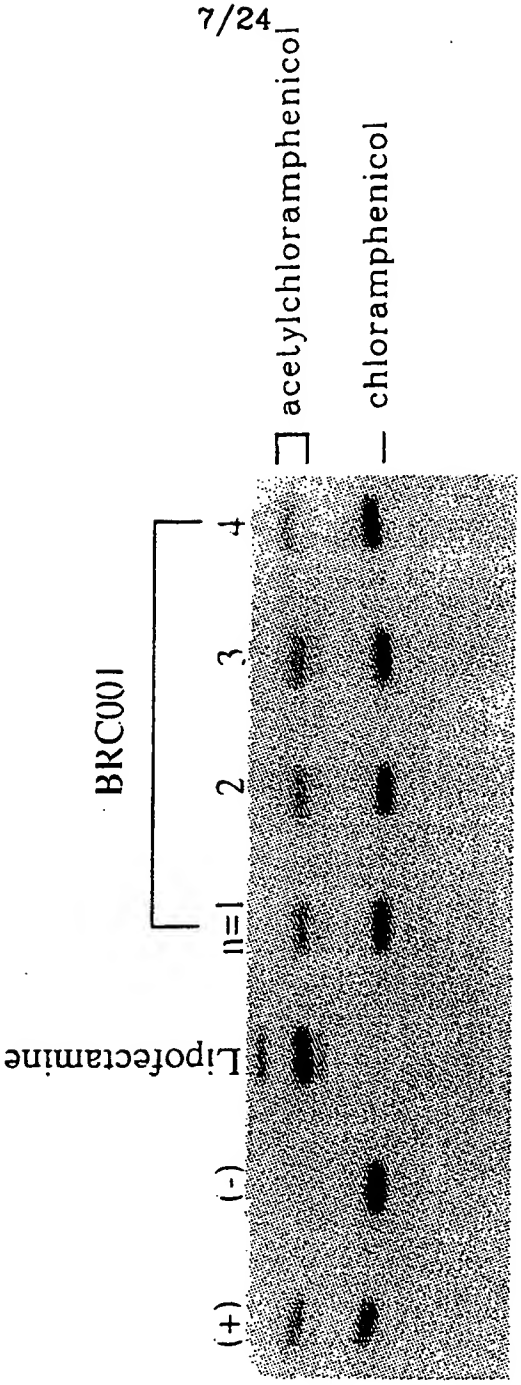

acetylchloramphenicol
chloramphenicol
BRC001
n=1
2
3
4
Lipofectamine
(+)
(-)

FIG.8

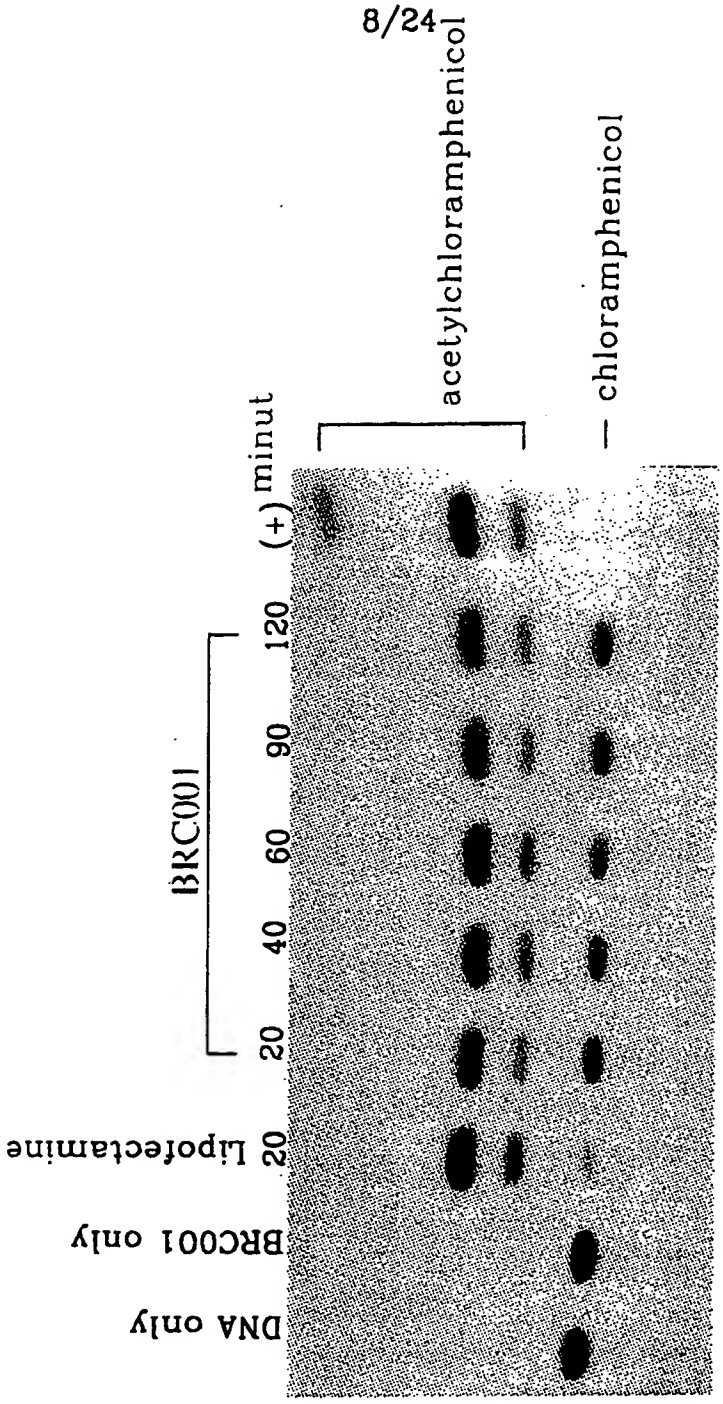

acetylchloramphenicol
chloramphenicol
(+) minut
120
90
60
40
20
BRC001
20
Lipofectamine
BRC001 only
DNA only

FIG.9A

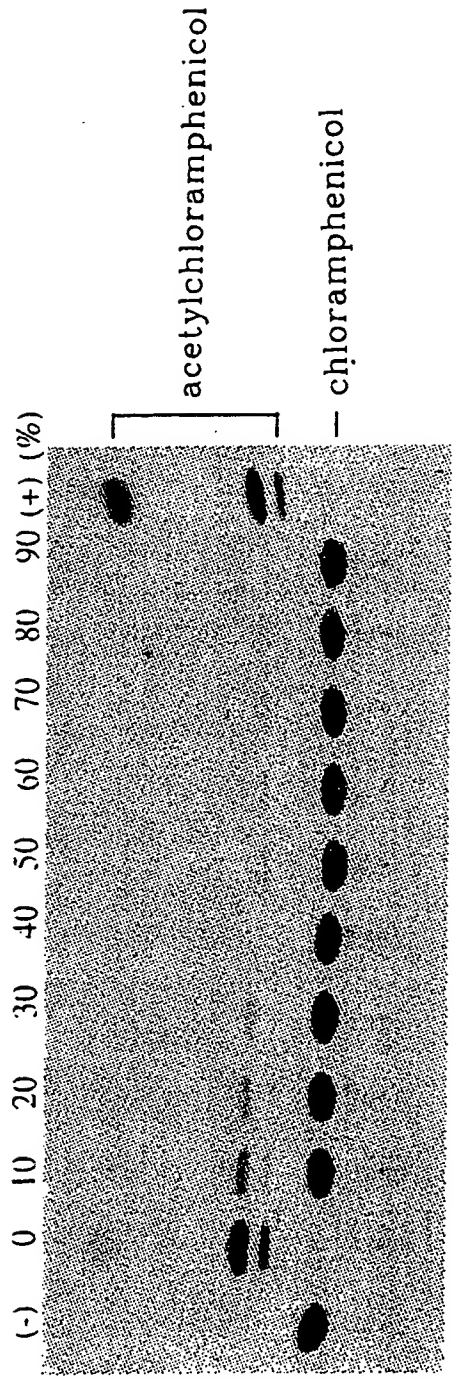

acetylchloramphenicol
chloramphenicol
(-)
0
10
20
30
40
50
60
70
80
90 (+) (%)

FIG.9B

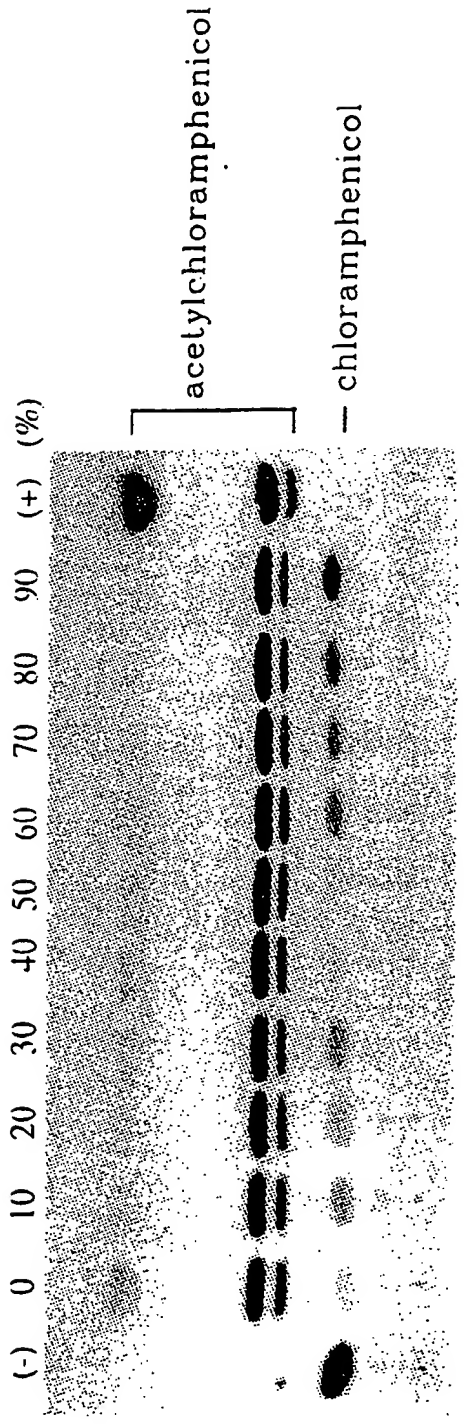

acetylchloramphenicol
chloramphenicol
(%)
(-) 0 10 20 30 40 50 60 70 80 90 (+)

FIG. 10A

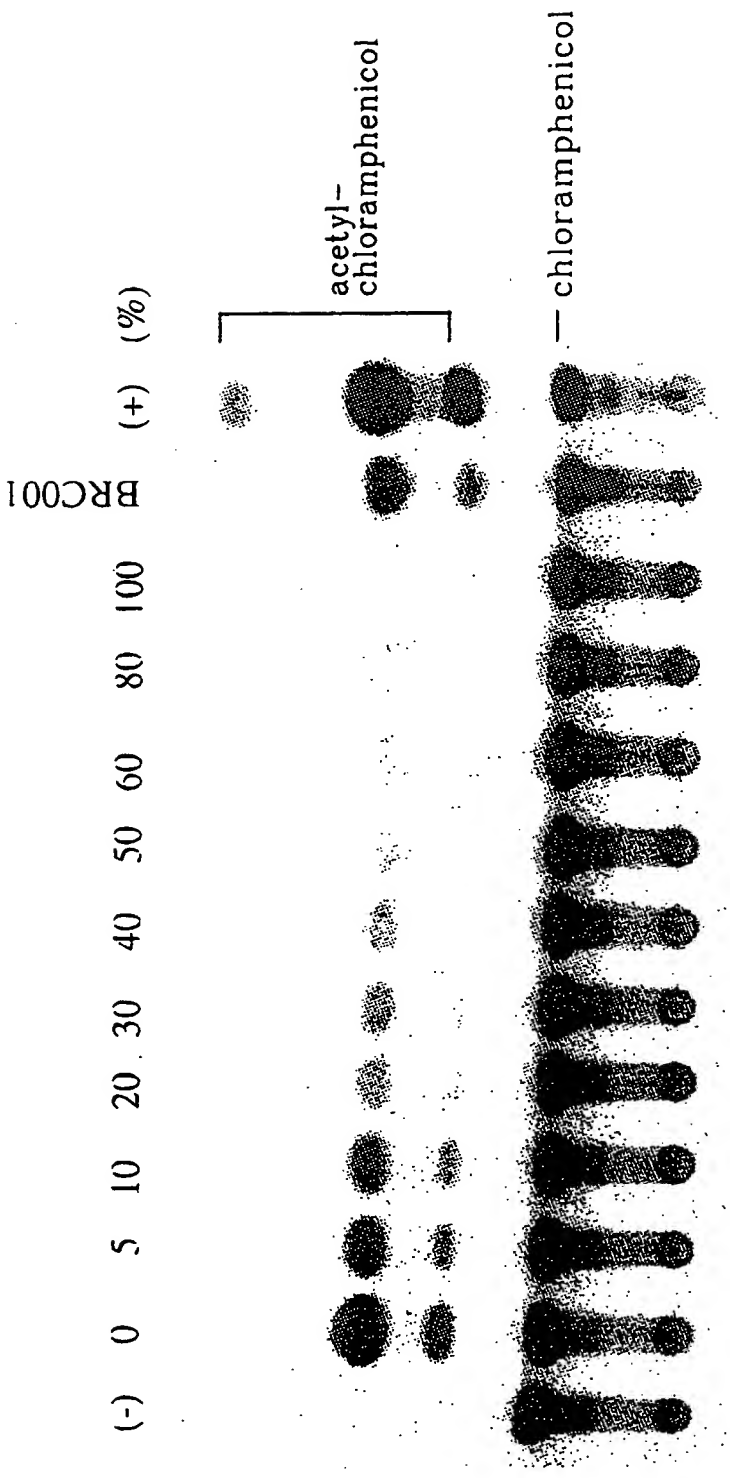

acetyl-
chloramphenicol
chloramphenicol
(%)
(+)
BRC001
100
80
60
50
40
30
20
10
5
0
(-)

FIG.10B

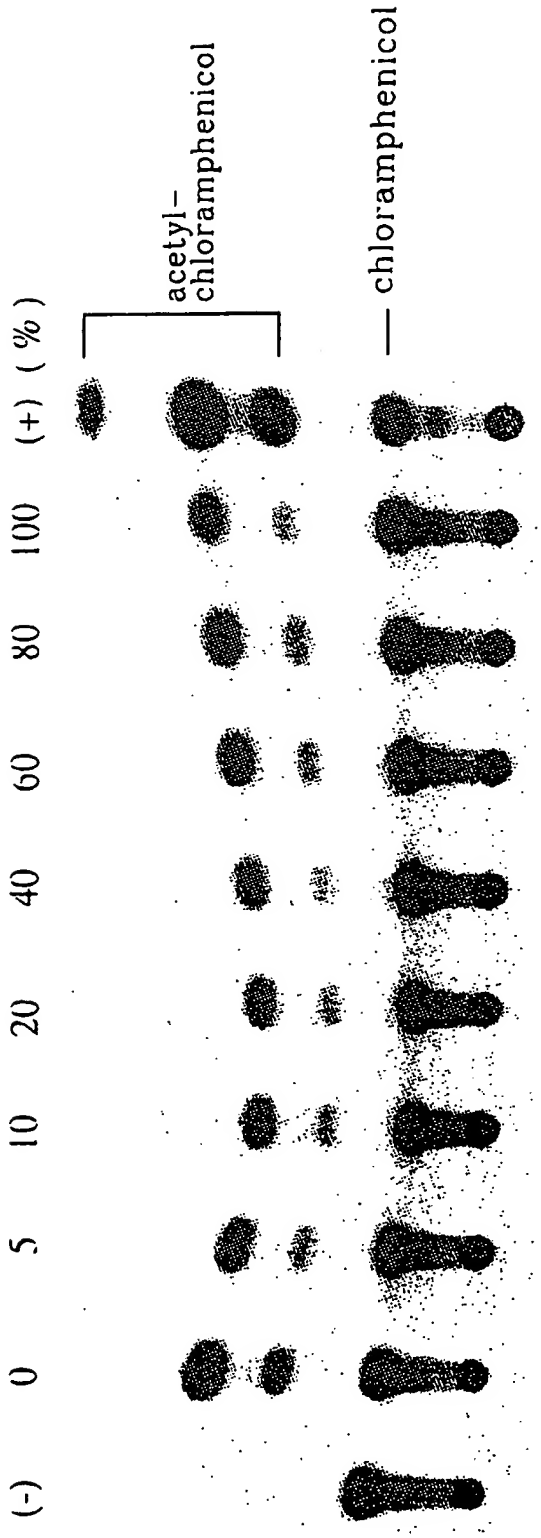

acetyl-
chloramphenicol
chloramphenicol
(+) (%)
100
80
60
40
20
10
5
0
(-)

FIG.11

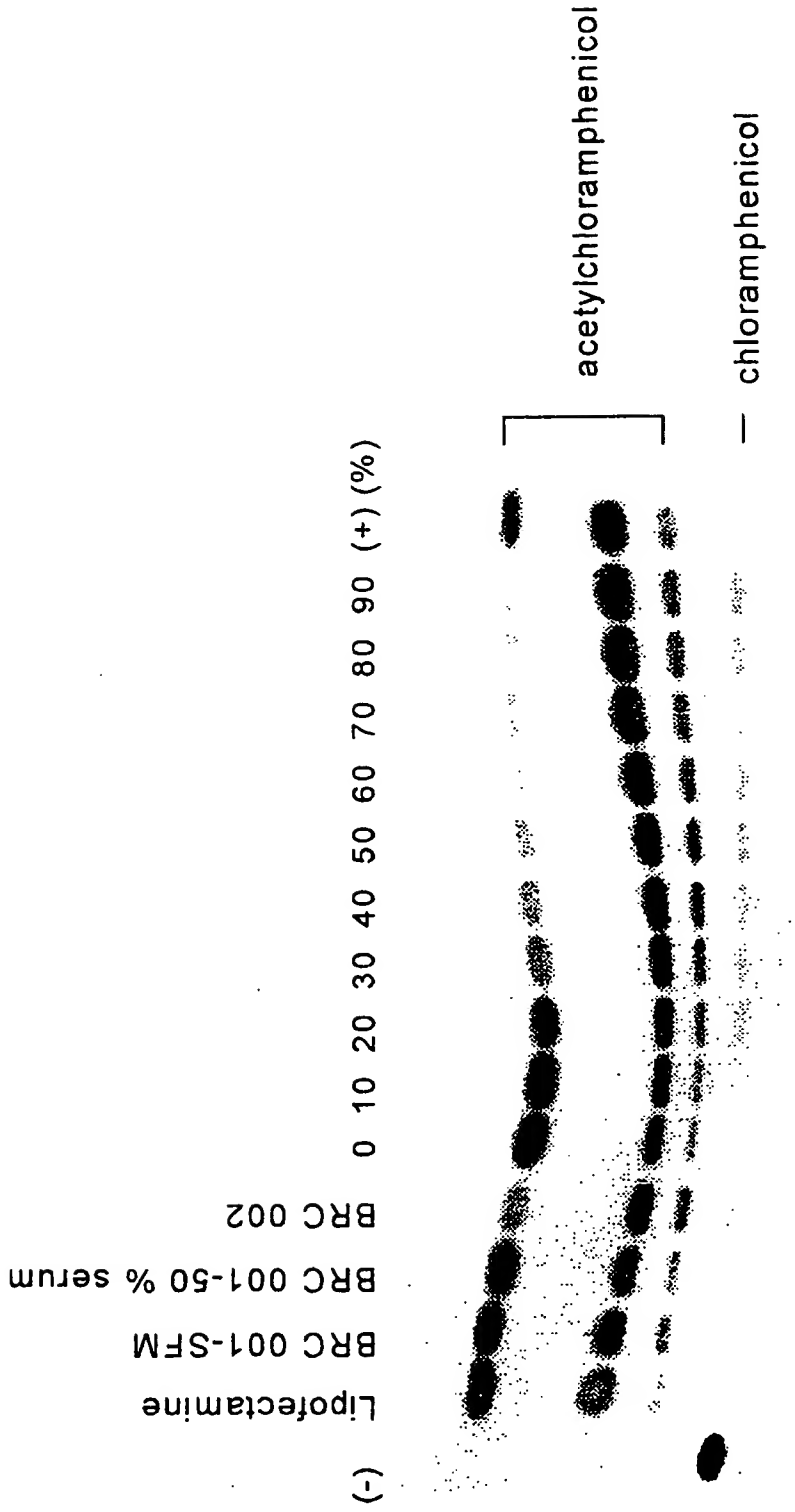

acetylchloramphenicol
chloramphenicol
(-)
Lipofectamine
BRC 001-SFM
BRC 001-50 % serum
BRC 002
0 10 20 30 40 50 60 70 80 90 (+) (%)

FIG. 12

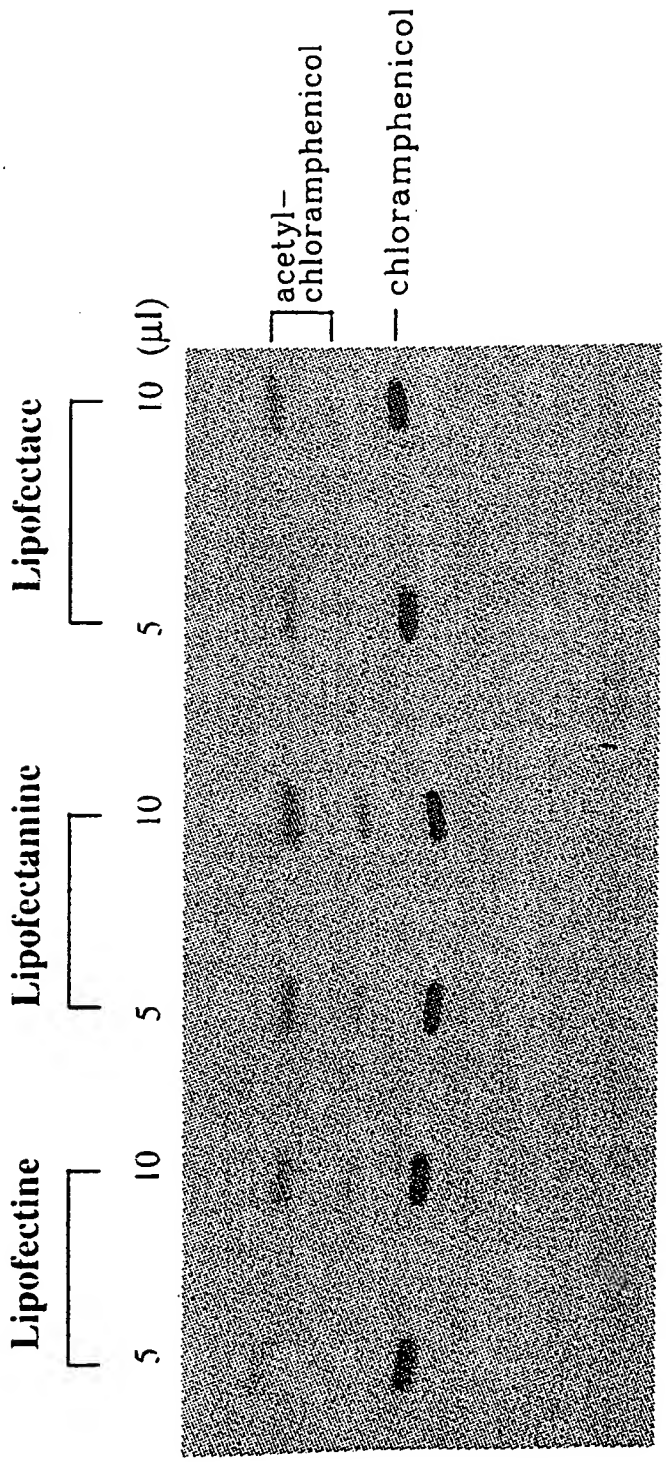

Lipofectine
5
10
Lipofectamine
5
10
Lipofectace
5
10 (μl)
acetyl-chloramphenicol
chloramphenicol

FIG. 13

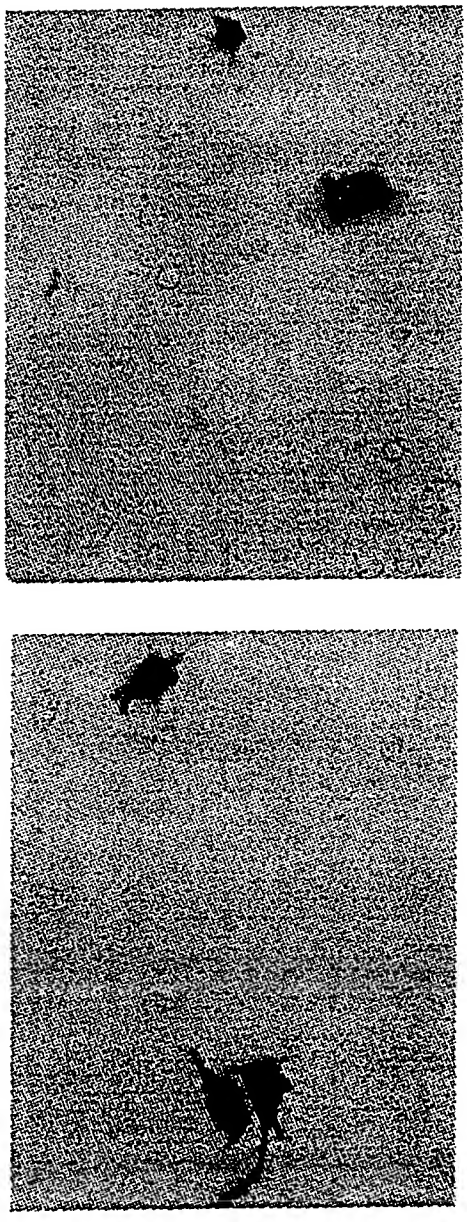


FIG. 14

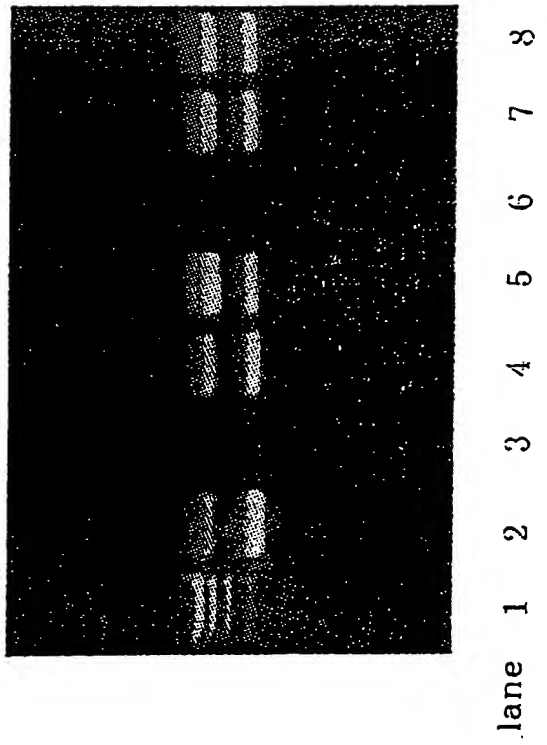


FIG. 15

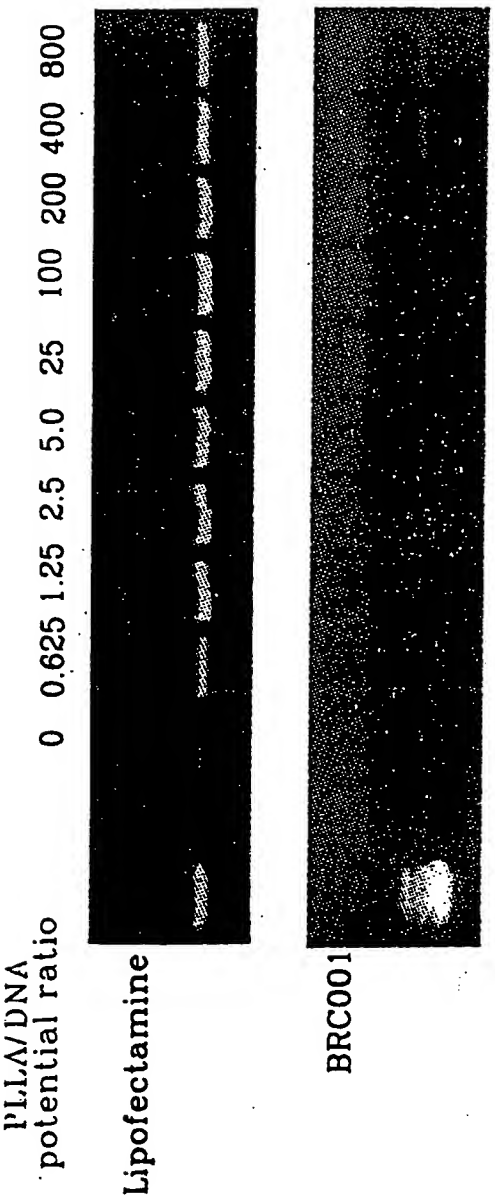

PLLA/DNA
potential ratio
0 0.625 1.25 2.5 5.0 25 100 200 400 800
Lipofectamine
BRC001

FIG. 16

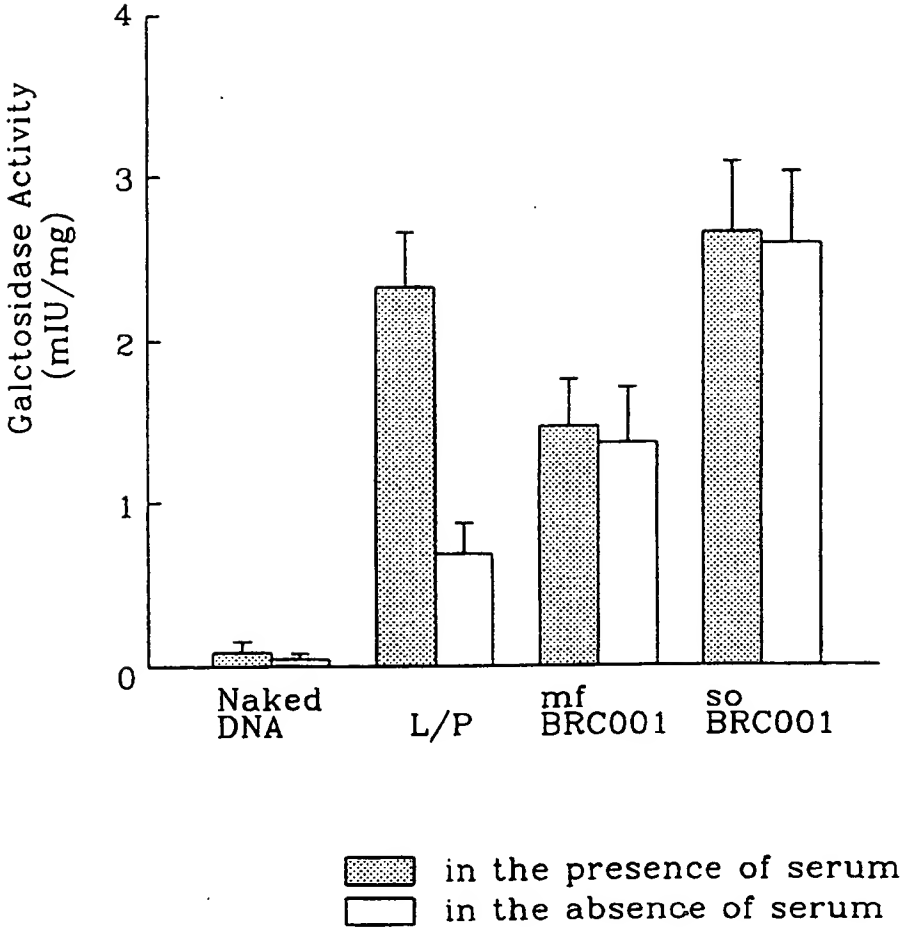

Galctosidase Activity
(mIU/mg)
4
3
2
1
0
Naked DNA
L/P
mf BRC001
so BRC001
in the presence of serum
in the absence of serum

FIG. 17

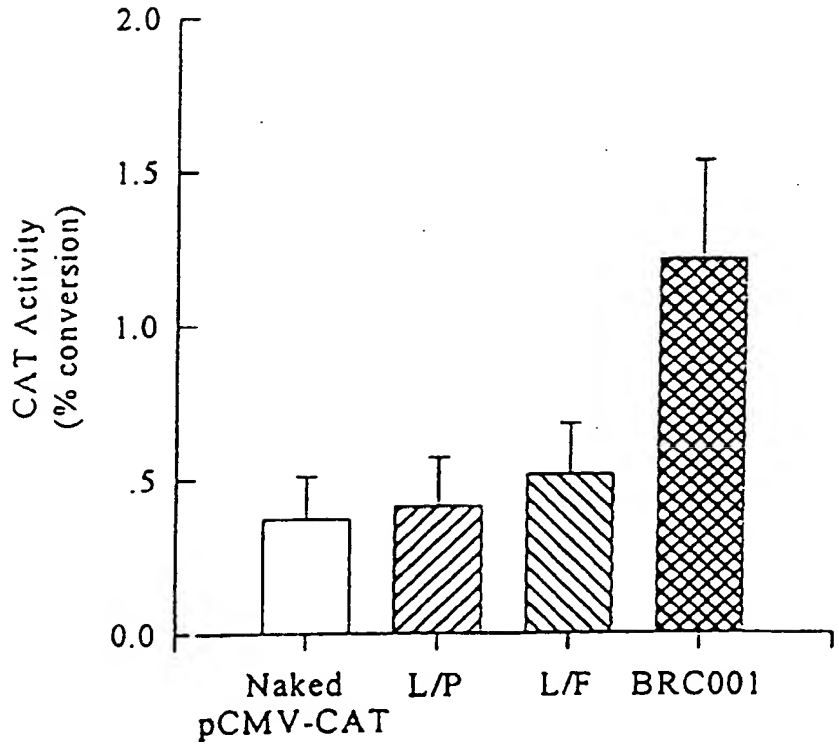

2.0
1.5
1.0
.5
0.0
CAT Activity
(% conversion)
Naked
pCMV-CAT
L/P
L/F
BRC001

FIG.18

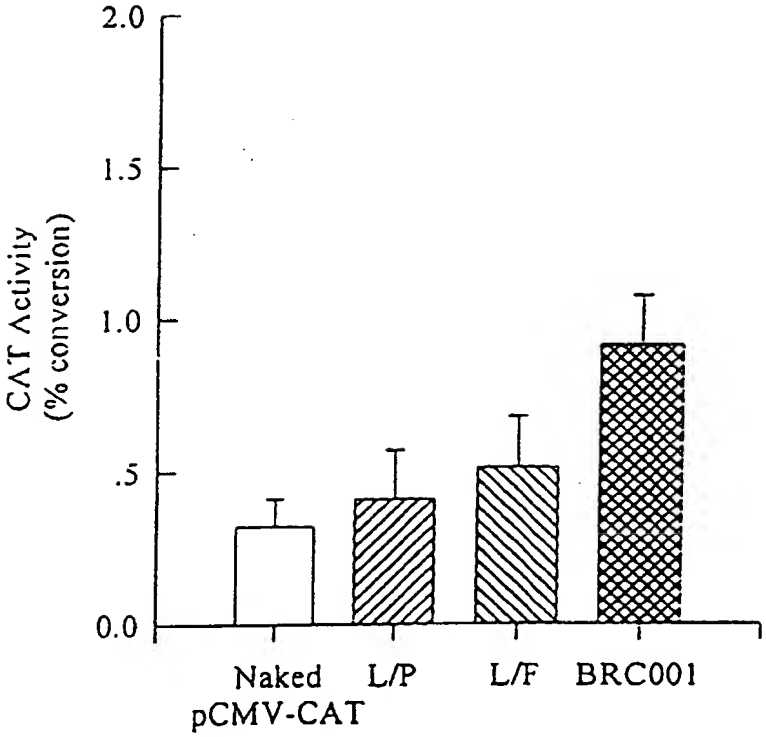

2.0
1.5
1.0
.5
0.0
CAT Activity
(% conversion)
Naked
pCMV-CAT
L/P
L/F
BRC001

FIG.19

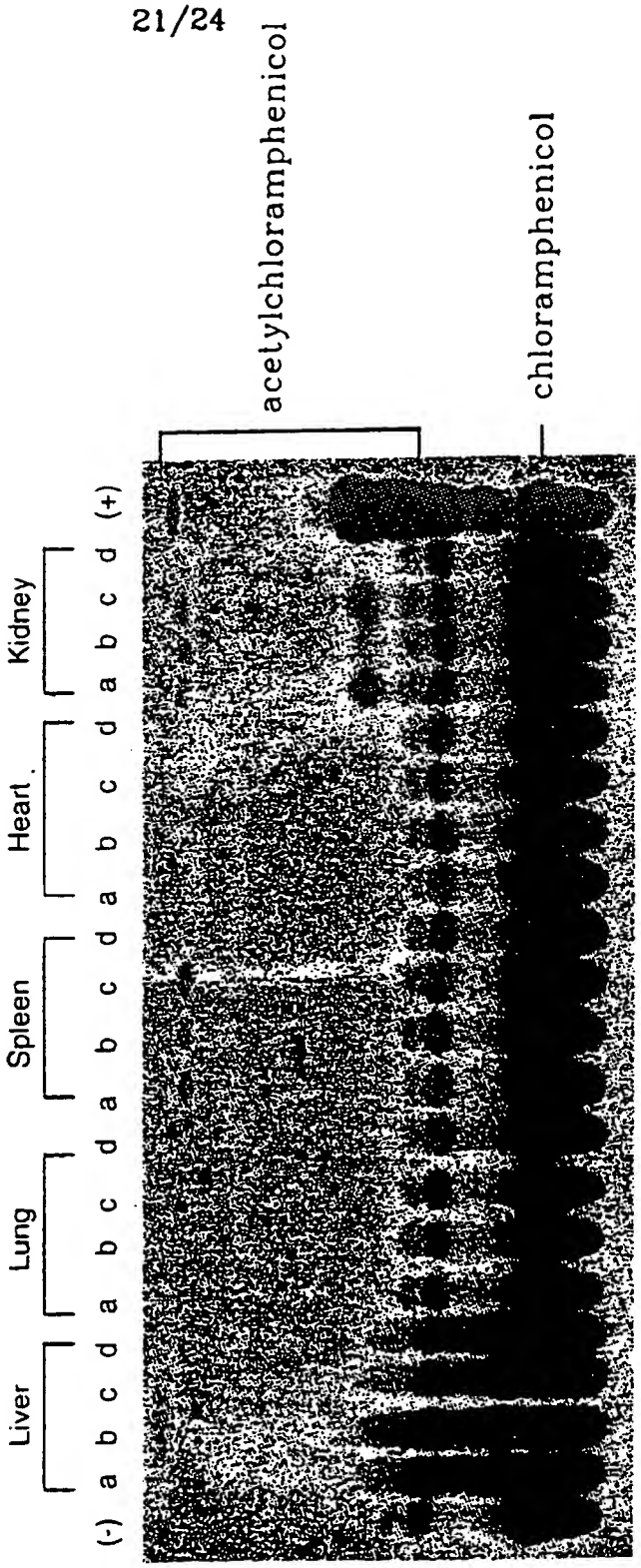

acetylchloramphenicol
chloramphenicol
Liver
Lung
Spleen
Heart
Kidney
(-) a b c d a b c d a b c d a b c d (+)

FIG.20

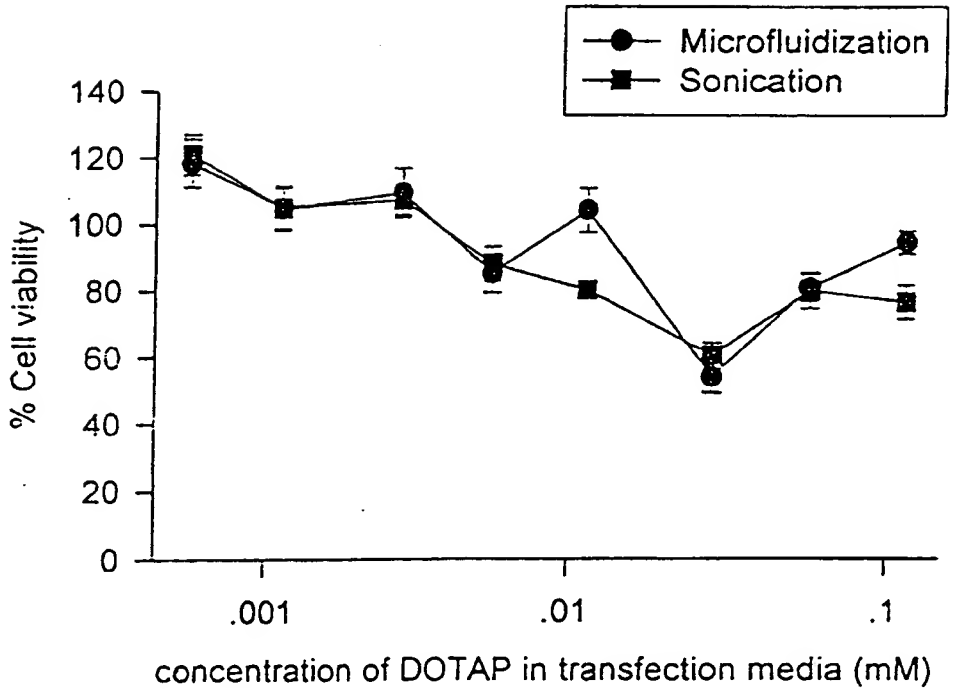

Microfluidization
Sonication
140
120
100
80
60
40
20
0
% Cell viability
.001
.01
.1
concentration of DOTAP in transfection media (mM)

FIG.21A

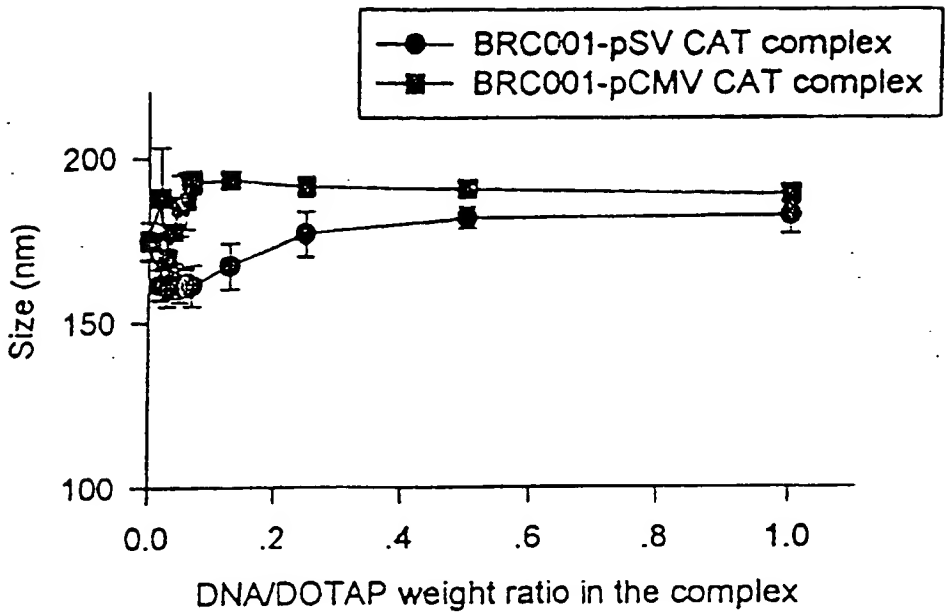



FIG.21B

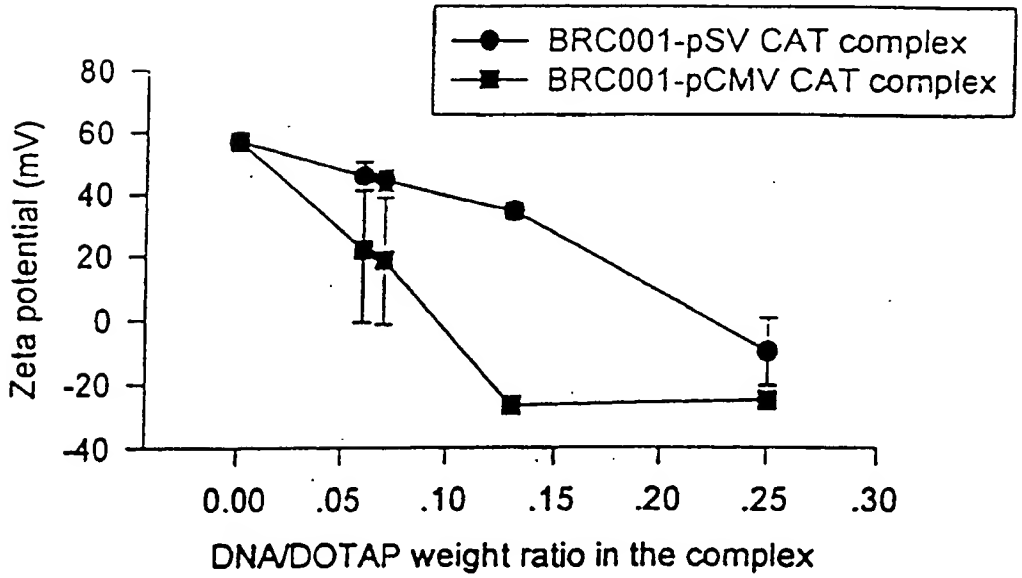


FIG.22A

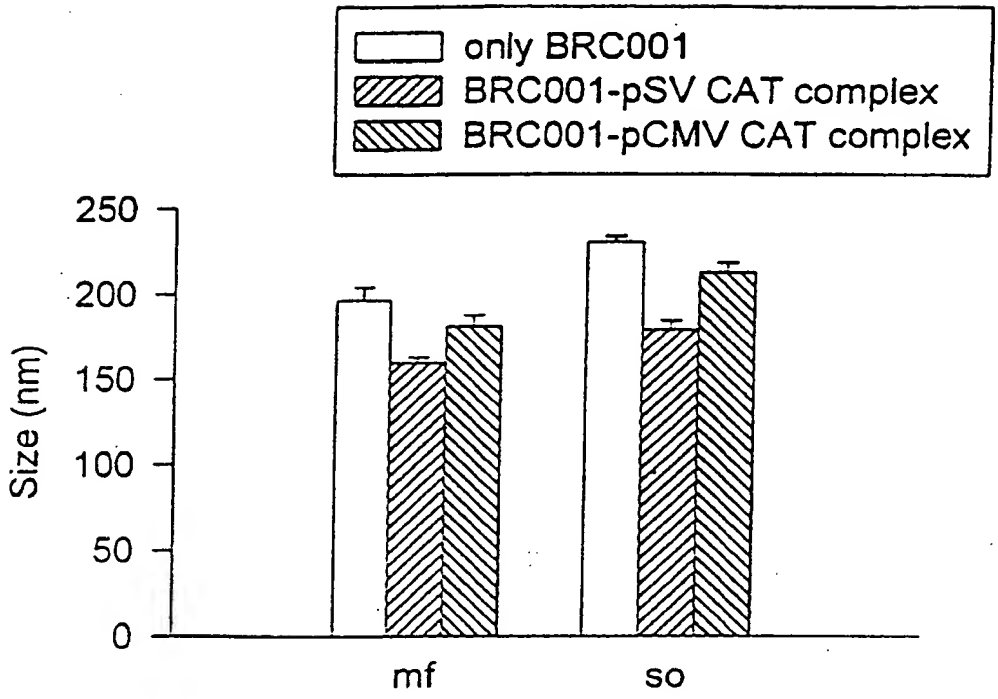
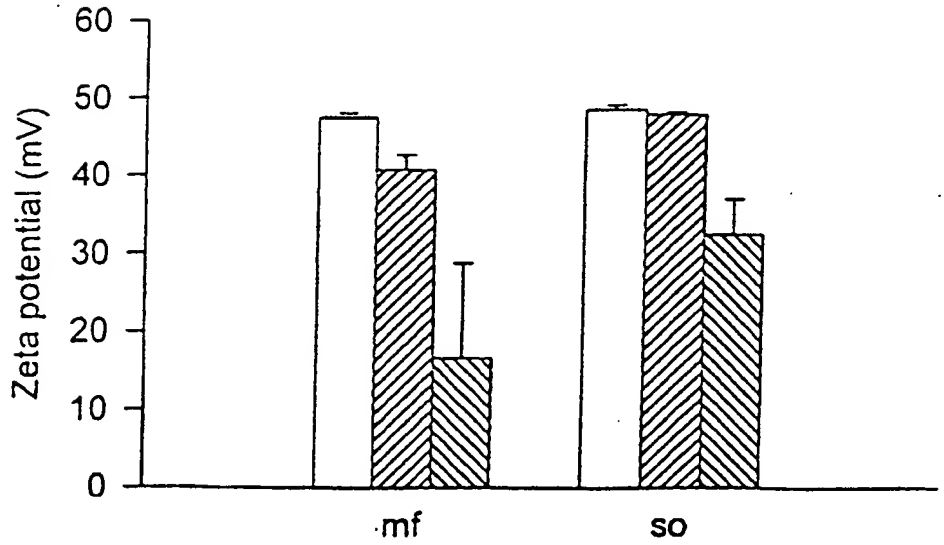



FIG.22B



# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00020

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 P 7/64; C 12 N 15/00; A 61 K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 P 7/64; C 12 N 15/00; A 61 K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DD 238 720 A1 (AKADEMIE DER WISSENSCHAFTEN DER DDR) 03 September 1986 (03.09.86), claims 1,6. | 1,10 |
| A | WO 95/17 953 A1 (FMC CORPORATION) 06 July 1995 (06.07.95), claims 1,9,12. | 1 |
| A | WO 96/08 235 A1 (DEPOTECH CORPORATION) 21 March 1996 (21.03.96), claim 1. | 1 |
| A | US 5 332 595 A (GAONKAR) 26 July 1994 (26.07.94), claim 1. | 1 |

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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 April 1998 (15.04.98) | 29 April 1998 (29.04.98) |
| Name and mailing address of the ISA/ AT<br>AUSTRIAN PATENT OFFICE<br>Kohlmarkt 8-10<br>A-1014 Vienna<br>Facsimile No. 1/53424/535 | Authorized officer<br>Wolf<br>Telephone No. 1/53424/436 |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00020

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 16
because ~~they~~ it relate to subject matter not required to be searched by this Authority, namely:

Treatment of the human or animal body by therapy (in vivo process), see Rule 39.1 (iv) PCT.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**
Information on patent family members

International application No.
PCT/KR 98/00020

| In Recherchenbericht angeführtes Patentdokument<br>Patent document cited in search report<br>Document de brevet cité dans le rapport de recherche | Datum der Veröffentlichung<br>Publication date<br>Date de publication | Mitglied(er) der Patentfamilie<br>Patent family member(s)<br>Membre(s) de la famille de brevets | Datum der Veröffentlichung<br>Publication date<br>Date de publication |
|---|---|---|---|
| DD A1 238720 | 03-09-86 | keine - none - rien | |
| WO A1 9517953 | 06-07-95 | AU A1 14073/95 | 17-07-95 |
| | | EP A1 737097 | 16-10-96 |
| | | EP A4 737097 | 09-04-97 |
| | | JP T2 9501100 | 04-02-97 |
| WO A1 9608235 | 21-03-96 | AU A1 35115/95 | 29-03-96 |
| | | CA AA 2199004 | 21-03-96 |
| | | EP A1 781123 | 02-07-97 |
| | | FI A0 971037 | 12-03-97 |
| | | FI A 971037 | 12-05-97 |
| | | JP T2 10502667 | 10-03-98 |
| | | NO A0 971149 | 12-03-97 |
| | | NO A 971149 | 13-05-97 |
| US A 5332595 | 26-07-94 | keine - none - rien | |